

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
20 November 2003 (20.11.2003)

PCT

(10) International Publication Number
WO 03/094974 A1

(51) International Patent Classification⁷: A61K 47/48 (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(21) International Application Number: PCT/GB03/01985 (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(22) International Filing Date: 8 May 2003 (08.05.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data: 0210538.5 8 May 2002 (08.05.2002) GB

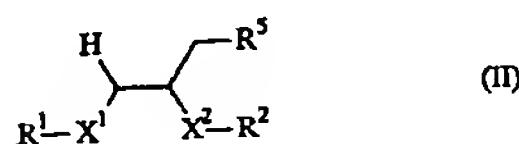
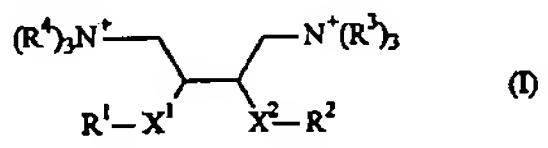
(71) Applicant (for all designated States except US): UNIVERSITY COLLEGE LONDON [GB/GB]; Gower Street, London WC1E 9BT (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): HURLEY, Christopher, Antony [GB/GB]; 2 Wensleydale, Hemel Hempstead, Hertfordshire HP2 5TF (GB). HAILES, Helen, Claire [GB/GB]; 20 Gordon Street, London WC1H 0AJ (GB). TABOR, Alethea, Bernice [GB/GB]; 20 Gordon Street, London WC1H 0AJ (GB). HART, Stephen, Lewis [GB/GB]; 30 Guildford Street, London WC1N 1EH (GB).

(74) Agent: WOODS, Geoffrey, Corlett; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5JJ (GB).

(54) Title: COMPLEXES FOR THE DELIVERY OF BIOLOGICALLY-ACTIVE MATERIAL TO CELLS



WO 03/094974 A1

(57) Abstract: A complex suitable for delivery of a biologically-active material to a cell, which complex comprises: (i) a lipid of general formula (I) or (II): wherein: -X¹ and X² are the same or different and are selected from -O-CH₂- and -O-C(O)-; -R¹ and R² are the same or different and are straight or branched, saturated or unsaturated C₇ to C₂₄ hydrocarbyl groups which are unsubstituted or substituted by one or more substituents selected from hydroxy, halogen and OR', wherein R' is a C₁ to C₆ hydrocarbyl group; -Each R³ and each R⁴ is the same or different and is a straight or branched, saturated or unsaturated C₁ to C₁₀ hydrocarbyl group which is unsubstituted or substituted by one or more substituents selected from hydroxy, halogen, -OR', -C(O)OH, -CN, -NR'R", and -C(O)R" wherein R' and R" are the same or different and are C₁ to C₆ hydrocarbyl; (Formula II) Wherein: X¹ and X² are the same or different and are as defined above; R¹ and R² are the same or different and are as defined above; R⁵ is -N[⊕](R³)₂-R⁶ wherein each R³ is the same or different and is as defined above and R⁶ is either: (a) (Formula A) wherein each Y is the same or different and is -N[⊕](R⁴)₂-; wherein R⁴ is as defined above, each A is the same or different and is a C₁ to C₂₀ alkylene group which is unsubstituted or substituted by one or more substituents selected from hydroxy, halogen, -OR', -C(O)OH, -CN, -NR'R", and -C(O)R" wherein R' and R" are the same or different and are C₁ to C₆ hydrocarbyl, n is from 1 to 10, and R⁴ is as defined above; or (b)-(Formula B) wherein each B is the same or different and is a C₁ to C₁₀ alkylene group which is unsubstituted or substituted by one or more substituents selected from hydroxy, halogen, -OR', -C(O)OH, -CN, -NR'R" and -C(O)R" wherein R' and R" are the same or different and are C₁ to C₆ hydrocarbyl, m is from 1 to 10, and Q is selected from N[⊕](R³)₃, OH, OR', OC(O)R' and halogen, wherein R³ and R' are as defined above; and (ii) A biologically-active material. Such complexes may be used to deliver biologically active material to a cell, for example in gene therapy and vaccination.

COMPLEXES FOR THE DELIVERY OF BIOLOGICALLY-ACTIVE MATERIAL TO CELLS

5 **Field of the invention**

The present invention relates to a complex suitable for delivery of a biologically-active material, for example nucleic acids, proteins and small molecules, to a cell. The invention also relates to the use of such complexes in the delivery of biologically-active material to a cell, for example in prophylaxis, treatment and 10 vaccination. Further, the invention relates to lipids which may be used in the complexes of the invention.

Background to the invention

Gene delivery for therapy or other purposes is of course well-known, 15 particularly for the treatment of diseases such as cystic fibrosis and certain cancers. The term refers to the delivery into a cell of a gene or part of a gene to correct some deficiency. In the present specification the term is used also to refer to any introduction of nucleic acid material into target cells, and includes gene vaccination and the *in vitro* production of commercially-useful proteins in so-called cell factories.

20 Cell delivery systems fall into three broad classes, namely those that involve direct injection of naked DNA, those that make use of viruses or altered viruses and those that make use of non-viral delivery agents. Each has its advantages and disadvantages. Although viruses as delivery agents have the advantages of high efficiency and high cell selectivity, they have the disadvantages of toxicity, 25 production of inflammatory responses and difficulty in dealing with large DNA fragments. The present invention, in making use of lipids, can overcome these problems.

Cationic lipids for use in gene delivery were developed by Felgner in the late 30 1980s, and reported in Proc. Natl. Acad. Sci. USA 84, 7413-7417, 1987. A recent patent to Felgner *et al.* that may be referred to is US 5264618. The disclosure of each of these documents is incorporated herein by reference. Felgner developed the now

-2-

commercially-available cationic liposome known by the trade mark "Lipofectin" which consists of the cytofectin, DOTMA and the neutral lipid DOPE in a 1:1 ratio. Various other cationic liposome formulations have since been devised, most of which combine a synthetic cationic cytofectin and a neutral lipid. Reference may also be 5 made to the use of targeting proteins, for example as used in so-called LID vectors. These vectors are three component vectors consisting of an integrin binding peptide, a lipid or lipid mixture such as Lipofectin and DNA. Specificity results from the targeting of the integrin, and transfection efficiencies comparable to some adenoviral vectors can be achieved (Hart *et al.*, Hum. Gene Ther. 9, 1037-47, 1998; Harbottle *et* 10 *al.* Hum. Gene Ther. 9, 575-85, 1998; and Jenkins *et al.* Gene Therapy 7, 393-400, 2000, the disclosures of which are incorporated herein by reference).

Cytofectins are positively charged molecules having a cationic head group attached via some spacer to a hydrophobic tail. In addition to the DOTMA analogues, there may be mentioned complex alkylamine/alkylamides, cholesterol 15 derivatives, and synthetic derivatives of dipalmitol, phosphatidyl-ethanolamine, glutamate, imidazole and phosphonate. A review of these materials, and of the mechanisms by which they operate, may be found in Angew. Chem. Int. Ed. 37, 1768-1785, 1998, the disclosure of which is incorporated herein by reference.

Various mechanisms have been suggested. An early suggestion was that 20 membrane fusion between liposome and cell membrane occurs. More recently, endocytosis of intact complexes has been proposed. Complexes formed between the nucleic acid and the lipid become attached to the cell surface, and then enter by endocytosis. They then remain localised within a vesicle or endosome. Migration to the nucleus may then occur some time later, where endosome fusion occurs allowing 25 the complexes to coalesce.

The use of lipids, rather than viruses, for this purpose can result in lower toxicity, reduced cost, reasonably efficient targeting, and the ability to deal with large fragments of nucleic acid material. Unfortunately, lower transfection efficiencies have been noted.

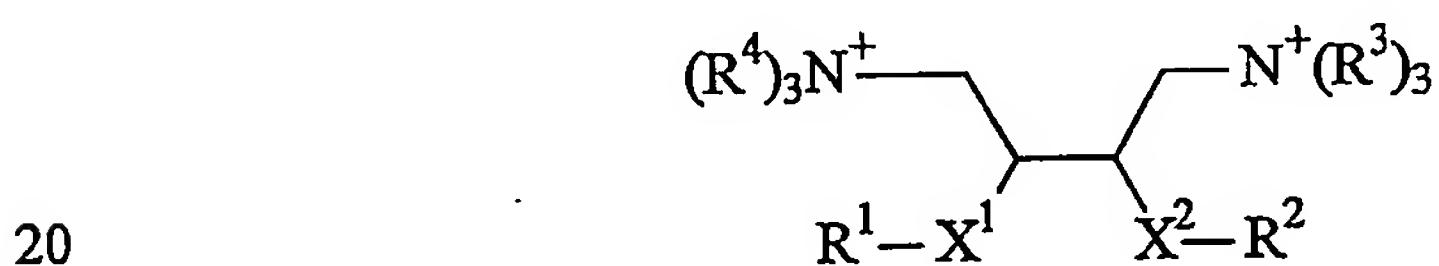
Summary of the invention

We have considered the factors that affect transfection, including phase transition temperatures, lipid chain length, the presence or absence of unsaturation in the lipid, the size of the lipid head group, and the charge on the lipid. From these 5 considerations we have designed new lipids that can improve transfection efficiencies. The nucleic acid must be delivered in a form in which it will be taken up, or internalised, by the target cell and allow it to be expressed properly. Also, the nucleic acid must, in general, be protected against certain cellular enzymes such as 10 nucleases, and for *in vivo* applications have suitable stability to serum. Thus, one must consider both internalization and protection when designing a lipid vector.

We have devised certain new dicationic lipids and also certain new PEG-based lipids incorporating a spacer between cationic centres

According to the invention there is thus provided a complex suitable for 15 delivery of a biologically-active material to a cell, which complex comprises:

(i) a lipid of general formula (I) or (II):



(I)

wherein:

25

- X^1 and X^2 are the same or different and are selected from $-\text{O}-\text{CH}_2-$, and $-\text{O}-\text{C}(\text{O})-$;
- R^1 and R^2 are the same or different and are straight or branched, saturated or unsaturated C_7 to C_{24} hydrocarbyl groups which are unsubstituted or substituted by one or more substituents selected from hydroxy, halogen and

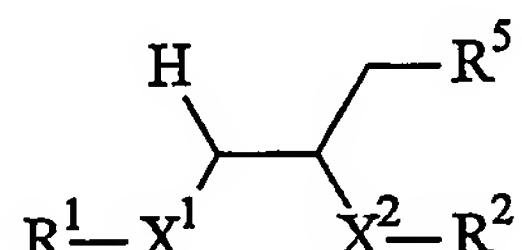
30

- OR' , wherein R' is a C_1 to C_6 hydrocarbyl group;

-4-

- each R³ and each R⁴ is the same or different and is a straight or branched, saturated or unsaturated C₁ to C₁₀ hydrocarbyl group which is unsubstituted or substituted by one or more substituents selected from hydroxy, halogen, -OR', -C(O)OH, -CN, -NR'R'', and -C(O)R'' wherein R' and R'' are the same or different and are C₁ to C₆ hydrocarbyl;

5



10

wherein:

- X¹ and X² are the same or different and are as defined above;
- R¹ and R² are the same or different and are as defined above;
- 15 - R⁵ is -N^o(R³)₂-R⁶ wherein each R³ is the same or different and is as defined above and R⁶ is either
 - (a) {A-Y}_nR⁴ wherein each Y is the same or different and is -N^o(R⁴)₂-, wherein R⁴ is as defined above,
- 20 each A is the same or different and is a C₁ to C₂₀ alkylene group which is unsubstituted or substituted by one or more substituents selected from hydroxy, halogen, -OR', -C(O)OH, -CN, -NR'R'', and -C(O)R'' wherein R' and R'' are the same or different and are C₁ to C₆ hydrocarbyl, n is from 1 to 10, and
- 25 R⁴ is as defined above; or
 - (b) {B-O}_mB-Q wherein each B is the same or different and is a C₁ to C₁₀ alkylene group which is unsubstituted or substituted by one or more substituents selected from hydroxy, halogen, -OR', -C(O)OH, -CN, -NR'R'' and -C(O)R'' wherein R' and R'' are the same or different and are C₁ to C₆ hydrocarbyl,

30

-5-

m is from 1 to 10, and

Q is selected from $N^+(R^3)_3$, OH, OR', OC(O)R' and halogen, wherein R³ and R' are as defined above; and

(ii) a biologically-active material.

5 The invention also provides:

– a process for the preparation of a complex of the invention, which method comprises: admixing (i) a lipid of formula (I) or (II) as defined above; and (ii) a biologically-active material;

– a complex obtainable by such a process;

10 – a mixture comprising:

(i) a lipid of formula (I) or (II) as defined above; and

(ii) (a) an integrin-binding peptide; and/or

(b) a polycationic component; and/or

(c) a neutral lipid;

15 – a process for the preparation of a complex of the invention, which process comprises admixing a mixture of the invention with a biologically-active material;

– a method for transfecting a cell with a biologically-active material, which method comprises contacting the cell *in vivo*, *in vitro* or *ex vivo* with a

20 complex of the invention;

– a method for the expression of a nucleic acid in a cell, which method comprises transfecting the cell with a complex of the invention using the method set out above under conditions to provide for expression of the nucleic acid component of the complex;

25 – a method for the preparation of a polypeptide, which method comprises:

(a) transfecting a cell with a complex of the invention using the method set out above under conditions to provide for expression of the polypeptide encoded by the nucleic acid component of the complex; and

(b) recovering the expressed polypeptide;

30 – use of a complex of the invention in transfecting a cell, in expressing a

-6-

nucleic acid in a cell or in the preparation of a polypeptide;

- a pharmaceutical composition comprising a complex of the invention and a pharmaceutically-acceptable carrier, diluent or excipient;
- a complex of the invention for use in a method of prophylaxis or treatment of the human or animal body by therapy;
- use of a complex of the invention in the manufacture of a medicament for use in the prophylaxis or treatment of a condition caused by or related to a genetic defect or modification;
- use of a complex of the invention in the manufacture of a medicament for use in the prophylaxis or treatment of a condition by an anti-sense nucleic acid or an iRNA;
- a method for the treatment of a condition caused by or related to a genetic defect or modification in a host, which method comprises administering to the host a therapeutically effective amount of a complex of the invention;
- a method for the treatment of a condition in a host by an anti-sense nucleic acid or an iRNA, which method comprises administering to the host a therapeutically effective amount of a complex of the invention;
- a vaccine comprising a complex of the invention and a pharmaceutically-acceptable carrier, diluent or excipient;
- a complex of the invention for use in a method of vaccinating a human or animal;
- use of a complex of the invention in the manufacture of a medicament for use in vaccinating a human or animal;
- a method for raising an immune response in a mammalian host, which comprises administering to the host a complex of the invention;
- a method for modifying a cell, which method comprises contacting the cell with a complex of the invention; and
- a lipid of formula (I) or (II) as defined above.

Detailed description of the invention

Throughout the present specification and the accompanying claims the words "comprise" and "include" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows.

The invention relates to complexes suitable for the delivery of a biologically-active material to a cell. The complexes are based on new lipids. The complexes of the invention may be used, for example, in gene therapy and vaccination. Gene 10 therapy may be carried out, for example, to correct a genetic defect of modification.

For the avoidance of doubt, in the general formulae (I) and (II) set out above, the orientation of the X^1 and X^2 moieties is such that the right hand side of the depicted moieties are attached to the group R^1 or R^2 .

The lipid of formula (I) or (II) is, of course, cationic, and will be associated 15 with one or more pharmaceutically acceptable anion. Examples of acceptable anions include anions of various mineral acids such as, for example, chloride, bromide, iodide, sulfate, nitrate, phosphate and anions of organic acids such as, for example, acetate, trifluoroacetate, maleate, fumarate, citrate, oxalate, succinate, tartrate, malate, mandelate, methanesulfonate and p-toluenesulfonate. Preferred anions are 20 chloride, bromide, iodide, sulphate, nitrate, acetate, maleate, oxalate and succinate. More preferred anions are bromide and iodide.

X^1 and X^2 are typically the same. X^1 and X^2 are preferably $-O-CH_2^-$.

Examples of unsaturated hydrocarbyl groups include alkenyl groups and 25 alkynyl groups. Preferred unsaturated hydrocarbyl groups are alkenyl groups which contain one or more, for example one or two, double bonds, each of which may be cis or trans. Typically, unsaturated hydrocarbyl groups are alkenyl groups which contain one or two cis double bonds. Typically, a said hydrocarbyl group is unsubstituted.

Typically, each R' and R'' is the same or different and is a C_1 to C_6 alkyl 30 group.

Preferably, R¹ and R² are the same or different and are straight or branched, saturated or unsaturated C₁₀ to C₂₂ hydrocarbyl groups which are unsubstituted or substituted as defined above. More preferably, R¹ and R² are the same or different and are straight or branched, saturated or unsaturated C₁₂ to C₂₀ hydrocarbyl groups which are unsubstituted or substituted as defined above. More preferably still, R¹ and R² are the same or different and are straight or branched, saturated or unsaturated C₁₆ to C₁₈ hydrocarbyl groups which are unsubstituted or substituted as defined above. Most preferably, R¹ and R² are the same or different and represent a palmitic, stearic, oleic, linoleic or linolenic residue. Most preferably, R¹ and R² are oleic residues [-10 (CH₂)₇CH=CH(CH₂)₇CH₃].

Typically, R¹ and R² are the same. Typically, R¹ and R² are unsubstituted or carry one, two or three substituents. Preferably, R¹ and R² are unsubstituted.

Typically, each R³ and each R⁴ is unsubstituted or substituted by one or more, for example one or two, substituents selected from hydroxy, -OR', -C(O)OH, -CN, -NR'R" and -C(O)R" wherein R' and R" are the same or different and are C₁ to C₆ hydrocarbyl.

Preferably, each R³ and each R⁴ are the same or different and are straight or branched, saturated or unsaturated C₁ to C₆ hydrocarbyl groups, for example C₁ to C₄ hydrocarbyl groups, which are unsubstituted or substituted by one or more substituents as defined above. Typically each R³ is the same. Typically each R⁴ is the same. Preferably each R³ and each R⁴ are the same.

Typically, R³ and R⁴ are C₁-C₁₀ alkyl groups, for example C₁-C₆ and C₁-C₄ alkyl groups. Preferably, R³ and R⁴ are methyl.

R³ and R⁴ are typically unsubstituted or carry one or two substituents. Preferred R³ and R⁴ substituents are selected from hydroxy and -OR' wherein R' is a C₁ to C₆ alkyl group. More preferably, R³ and R⁴ are unsubstituted.

Preferably, n is from 1 to 5. More preferably, n is from 1 to 2. Typically, n is 1.

Preferably, m is from 1 to 5. More preferably, m is from 1 to 3. Typically, m is 1 or 2.

-9-

Preferably, A is C₁ to C₁₀ alkylene, for example C₃, C₆ or C₁₀ alkylene, which is unsubstituted or substituted by one or more substituents as defined above. More preferably, A is C₂ to C₆ alkylene which is unsubstituted or substituted by one or more substituents as defined above. Yet more preferably, A is C₃, C₄ or C₅ alkylene which is unsubstituted or substituted by one or more substituents as defined above.

5 Most preferably, A is propylene which is unsubstituted or substituted by one or more substituents as defined above.

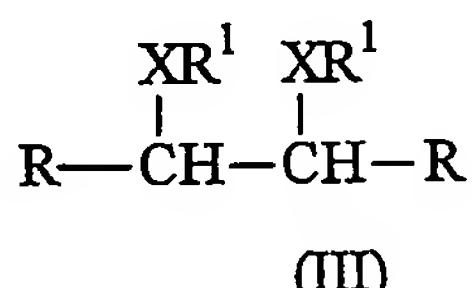
10 Typically, A is unsubstituted or carries one or two substituents. Preferred substituents for A are selected from hydroxy, halogen and -OR' wherein R' is a C₁ to C₆ alkyl group. More preferably, A is unsubstituted.

15 Preferably, B is C₁ to C₅ alkylene which is unsubstituted or substituted by one or more substituents as defined above. More preferably, B is C₂, C₃ or C₄ alkylene which is unsubstituted or substituted by one or more substituents as defined above. Most preferably, B is ethylene which is unsubstituted or substituted by one or more substituents as defined above.

Typically, B is unsubstituted or carries one or two substituents. Preferred substituents for B are selected from hydroxy, halogen and -OR' wherein R' is a C₁ to C₆ alkyl group. More preferably, B is unsubstituted.

Q is preferably -N°(R³)₃ or OH. Typically, Q is -N°Me₃ or OH.

20 The present invention also provides a composition including the structure (III):



25

wherein:

the Rs, which may be the same or different, are

- (a) H,
- (b) -CH₂-N°(R²)₂-CH₂-CH₂[Y-(CH₂)_p]_q-Z, or
- 30 (c) -CH₂-N°(R⁴)₃,

-10-

with the proviso that one R is H and the other is group (b); or both groups R are groups (c); and wherein

the Xs which may be the same or different, are OCH₂ or O-C(O);

the R¹s, which may be the same or different, are saturated or unsaturated, C7 to C23 chains;

5 the R²s, which may be the same or different, are C1 to C6 saturated or unsaturated chains;

Y is NH, CH₂, O or N(acetyl);

10 Z is O(C₁ to C₄), OC(O)R³, N⁺R₃⁴, OH, F, Cl, Br or I where R³ is C1 to C6 alkyl;

the R⁴s, which may be the same or different, are C1 to C6 chains;

n is from 2, 3 or 4; and

m is from 1 to 200 and where it is at least 2 the resulting repeating units may be the same or different.

15 In formula (III), we prefer that one R is hydrogen and the other is group (b); or that both groups R are groups (c). In the first of these cases the lipid may be PEG-based, and in the second case it is based on erythritol.

In formula (III), we prefer that m is less than or equal to 100, more preferably less than or equal to 50, especially less than or equal to 25, more especially less than 20 or equal to 12. Preferably m is at least 2.

In formula (III), we prefer that when Y is CH₂, Z is not OH and preferably not O(C1-C4).

25 Since formula (III) set out above is cationic, it will be accompanied by one or more appropriate non-toxic anions. Suitable anions include halide, particularly iodide.

In the erythritol-based lipid of the formula (III), we prefer that the two groups R are substantially identical and/or that the two groups R¹ are substantially identical. Where the Rs are substantially identical and the R¹s are substantially identical the overall structure will be substantially symmetrical.

30 Where the groups X in the formula (III) are OC, the linkages will of course be

ethers, and where the groups are O-C(O) the linkages will be esters. The ether structures are preferred because they seem to have greater activity.

The chain length of the tails R¹ in the formula (III) has been investigated. Including the carbon atom of the linkage X, we prefer from C10 to C22, and 5 preferably straight-chain. At present a value of about C16 or C18 seems to be optimum, although the precise value will depend on the application. Other possible values include C12, C14, and C20.

The tails may be saturated or unsaturated, and when unsaturated may contain 10 one or more double bonds, each of which may be cis or trans, and/or one or more triple bonds. Typical unsaturated structures include one or two cis double bonds. We have found higher transfection efficiencies with unsaturated lipids.

Groups R² in the formula (III) are preferably straight chain alkyl, although branched groups can be acceptable. We prefer C1, C2, C3 and C4. The same applies to group R³.

15 Although (when m is two or more) the repeating units [Y-(CH₂)-] in the formula (III) may all be the same, we prefer that Y be CH₂ in all but one of them, and therefore that it take on one of the other possibilities in only one of the repeating units. Of these other possibilities, O is at present preferred. The number of 20 methylene groups, n, is preferably 2, although other values for example 1, 3, 4, 5 and 6 may be desirable in some circumstances.

The lipids of the invention will contain one or more chiral centre. In particular, in the erythritol based lipids the two carbon atoms illustrated as such in the general formula given above will be chiral centres. Thus, there will be four isomers, unless the structure has a plane of symmetry between those carbon atoms in which 25 case there will be three isomers. For the avoidance of doubt, the chemical structures depicted herein are intended to embrace all stereoisomers of the compounds shown, including racemic and non-racemic mixtures and pure enantiomers and/or diastereoisomers.

30 We have found that the presence of two cationic charges, particularly the presence of a second quaternary ammonium centre and particularly when the

separation between the centres is as set out above, results in improved interaction between the lipid and the nucleic acid material. In general we prefer the spacer to be reasonably short, for example from C2 to C6, and preferably C3, C4 or C5. The intention is that complexes be formed that are capable of entering the target cells, 5 after which the nucleic acid can be released to be expressed in the cell nucleus or in some way to control or affect gene expression.

The lipids of the invention may be formulated with one or more other components as complexes which are suitable for use in the delivery of a biologically-active material to a cell. Typically, such complexes comprise a lipid of the invention 10 and a biologically-active material. Complexes of the invention may also comprise one or more of: an integrin-binding peptide; a polycationic component; and a neutral lipid.

Suitable biologically-active materials include nucleic acids, peptides and polypeptides, and small molecules. A biologically-active material is one which has a 15 biological effect when introduced into a cell or host, for example by stimulating an immune response or an inflammatory response, by exerting enzymatic activity or by complementing a mutation, etc. These particular biological activities are given merely by way of examples and are not to be taken as limiting.

The terms "nucleic acid" and "polynucleotide" are used interchangeably and 20 refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Non-limiting examples of polynucleotides include a gene, a gene fragment, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, cosmids, vectors, artificial chromosomes, isolated DNA 25 of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers.

Polynucleotides of the invention may include within them synthetic or 30 modified nucleotides. A number of different types of modification to polynucleotides are known in the art. Such modifications may be carried out in order to enhance the in vivo activity, lifespan, nuclease resistance or ability to enter cells. For example, phosphorothioate oligonucleotides may be used. Other

-13-

deoxynucleotide analogs include methylphosphonates, phosphoramidates, phosphorodithioates, N3'P5'-phosphoramidates and oligoribonucleotide phosphorothioates and their 2'-O-alkyl analogs and 2'-O-methylribonucleotide methylphosphonates.

5 Alternatively mixed backbone oligonucleotides (MBOs) may be used. MBOs contain segments of phosphothioate oligodeoxynucleotides and appropriately placed segments of modified oligodeoxy- or oligoribonucleotides. MBOs have segments of phosphorothioate linkages and other segments of other modified oligonucleotides, such as methylphosphonate, which is non-ionic, and very resistant to nucleases or 2'-
10 O-alkyloligonucleotides.

A DNA in a complex of the invention may be in the form of a linear molecule or a circular molecule, for example a plasmid or cosmid. Examples of linear DNA molecules include DNA in the form of a chromosome or a mini chromosome.

15 An RNA used in a complex of the invention may be polycistronic, i.e. may comprise more than one coding sequence, and therefore may comprise an internal ribosome entry site (IRES).

20 If a DNA for use in the invention comprises more than one DNA coding sequence, those coding sequences may be operably linked to independent control sequences. Alternatively, the coding sequences may be operably linked to common control sequences, in which case the coding sequences may be separated by an
25 (IRES).

An RNA in complex of the invention may be linear or circular, for example a replicon, in particular an alpha virus replicon. The RNA may be single stranded or double stranded. The RNA may be an mRNA. Suitable mRNAs will typically comprise a 5' cap and/or a 3' polyA tail. In addition, the length of the polyA tail may be modulated to regulate the stability of the mRNA within target cells and hence control the duration of transgene expression from the mRNA. Typically, a polyA tail will be up to about 300 residues in length, preferably from about 50 to about 90
30 residues in length.

A "gene" as used in the context of the present invention is a sequence of nucleotides in a genetic nucleic acid (chromosome, plasmid, etc.) with which a genetic function is associated. A gene is a hereditary unit, for example of an organism, comprising a polynucleotide sequence (e. g., a DNA sequence for mammals) that occupies a specific physical location (a "gene locus" or "genetic locus") within the genome of an organism. A gene can encode an expressed product, such as a polypeptide or a polynucleotide (e. g., tRNA). Alternatively, a gene may define a genomic location for a particular event/function, such as the binding of proteins and/or nucleic acids (e. g., phage attachment sites), wherein the gene does not encode an expressed product. Typically, a gene comprises coding sequences, such as polypeptide encoding sequences, and non-coding sequences, such as promoter sequences, poly-adenylation sequences, transcriptional regulatory sequences (e. g., enhancer sequences). Many eukaryotic genes have "exons" (coding sequences) interrupted by "introns" (non-coding sequences). In certain cases, a gene may share sequences with another gene (s) (e. g., overlapping genes).

A "coding sequence" or a sequence which "encodes" a selected polypeptide, is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences (or "control elements").

The boundaries of a coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from viral, prokaryotic or eukaryotic mRNA, genomic DNA sequences from viral or prokaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence may be located 3' to the coding sequence. Transcription and translation of coding sequences are typically regulated by "control elements", including, but not limited to, transcription promoters, transcription enhancer elements, transcription termination signals, polyadenylation sequences (located 3' to the translation stop codon), sequences for optimization of initiation of translation (located 5' to the coding sequence), and translation termination sequences.

-15-

A nucleic acid for use in the invention which comprises a coding sequence may be contained in an expression vector. A suitable expression vector comprises nucleotide sequences, for example a coding sequence encoding a desired peptide or polypeptide. Such expression vectors are routinely constructed in the art of molecular biology and may for example involve the use of plasmid or cosmid DNA and appropriate initiators, promoters, enhancers and other elements, such as for example polyadenylation signals which may be necessary, and which are positioned in the correct orientation, in order to allow for protein expression. Other suitable vectors would be apparent to persons skilled in the art. A nucleic acid suitable for use in the invention may also be inserted into a vector in an antisense orientation in order to provide for the production of antisense RNA.

5 A "promoter" is a nucleotide sequence which initiates transcription of a polypeptide-encoding polynucleotide. Promoters can include inducible promoters (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.), repressible promoters (where expression of a polynucleotide sequence operably linked to the promoter is repressed by an analyte, cofactor, regulatory protein, etc.), and constitutive promoters. In addition, such promoters can also have tissue specificity. It is intended that the term "promoter" or "control element" includes full-length promoter regions 10 and functional (e. g., controls transcription or translation) segments of these regions.

15 Thus, a polynucleotide, especially a coding, for use in a complex of the invention is operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence, such as a promoter, "operably linked" to a coding sequence is 20 positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequence. The control sequence will typically comprise a promoter and optionally also comprise other types of control sequence, for example an enhancer and/or terminator. An enhancer is any polynucleotide 25 30

sequence capable of increasing the level of transcription initiating from a promoter and may act on a *cis* or *trans* basis. A terminator is any polynucleotide sequence capable of promoting dissociation of an RNA polymerase from the said sequence.

5 A control sequence may be positioned 5', 3' or internal to (for example in an intron) a coding sequence. A coding sequence may be operably linked to more than one control sequence, for example two, three, four or five control sequences. Such multiple control sequences may be positioned, for example, entirely 5' to the coding sequence. However, more typically control sequences will be located both 5' and 3' to the coding sequence, with optional internal control sequences.

10 Control sequences may be derived from any suitable source and may be generated by recombinant techniques or synthetic means.

15 The vectors may be for example, plasmid, virus or phage vectors provided with a origin of replication, optionally a promoter for the expression of the desired polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a resistance gene for a fungal vector. Vectors may be used *in vitro*, for example for the production of DNA or RNA or used to transfect or transform a host cell, for example, a mammalian host cell. The vectors may also be adapted to be used *in vivo*, for example in a method of gene therapy or vaccination.

20 Suitable nucleic acids for use in a complex of the invention may be obtained from natural sources, or may be produced recombinantly or by chemical synthesis. They may be modified, for example, to comprise a sequence encoding a specific function, for example, a nuclear localisation sequence.

25 A nucleic acid in a complex of the invention may be selected for use in gene therapy, in gene vaccination, in anti-sense therapy or in therapy by interfering RNA. All of these uses may be generally referred to as gene therapy.

30 As has been set out above, appropriate transcriptional and translational control elements are generally provided. For gene therapy, the nucleic acid component is generally presented in the form of a nucleic acid insert in a plasmid or vector. In some cases, however, it is not necessary to incorporate the nucleic acid

-17-

component in a vector in order to achieve expression. For example, gene vaccination and anti-sense therapy can be achieved using a naked nucleic acid. The nucleic acid is generally DNA but RNA may be used in some cases, for example, in cancer vaccination.

5 The nucleic acid in a complex of the invention may be or may relate to a gene that is the target for particular gene therapy or may be a molecule that can function as a gene vaccine or as an anti-sense therapeutic agent. The nucleic acid may be or correspond to a complete full-length coding sequence or may be part of a coding sequence.

10 A nucleic acid may be selected to act via an antisense mechanism or via an RNA interference mechanism (RNAi). An antisense RNA may comprise a polynucleotide which has substantial complementarity to all or part of its target mRNA. A polynucleotide which has substantial sequence complementarity to all or part of its target mRNA is typically one which is capable of hybridizing to that 15 mRNA. If the RNA has substantial complementarity to a part of its target mRNA of, it generally has substantial complementarity to a contiguous set of nucleotides within that mRNA.

There are, generally speaking, two antisense approaches which may be used in the invention.

20 In one approach, a vector is used which allows for the expression of a polynucleotide which has substantial sequence complementarity to all or part of the target mRNA (i.e. a polynucleotide which can hybridize to that mRNA). This results in the formation of an RNA-RNA duplex which may result in the direct inhibition of translation and/or the destabilization of the target message, by rendering it 25 susceptibility to nucleases, for example. The vector will typically allow the expression of a polynucleotide which hybridizes to the ribosome binding region and/or the coding region of the target mRNA.

30 Alternatively, an oligonucleotide may be delivered which is capable of hybridizing to the target mRNA. Antisense oligonucleotides are postulated to inhibit target gene expression by interfering with one or more aspects of RNA metabolism,

for example processing, translation or metabolic turnover. Chemically modified oligonucleotides may be used and may enhance resistance to nucleases and/or cell permeability.

In the first approach, the vector is capable of expressing a polynucleotide which has substantial sequence complementarity to all of part of the target mRNA. Such a polynucleotide will be capable of hybridizing to the target mRNA. Typically, such a polynucleotide will be an RNA molecule. Such a polynucleotide may hybridize to all or part of the target mRNA. Generally, therefore the polynucleotide will be complementary to all of or part of such an mRNA. For example, the polynucleotide may be the exact complement of such an mRNA. However, absolute complementarity is not required and preferred polynucleotides which have sufficient complementarity (i.e. substantial complementarity) to form a duplex having a melting temperature of greater than 40°C under physiological conditions are particularly suitable for use in the present invention. The polynucleotide may be a polynucleotide which hybridises to the target mRNA under conditions of medium to high stringency, such as 0.03M sodium chloride and 0.03M sodium citrate at from about 50 to about 15 60 degrees centigrade.

It is preferred that the polynucleotide hybridizes to a coding region of the target mRNA. However, a polynucleotide may be employed which hybridises to all or part of the 5'- or 3'-untranslated region of such an mRNA. The polynucleotide will typically be at least 40, for example at least 60 or at least 80, nucleotides in length and up to 100, 200, 300, 400, 500, 600 or 700 nucleotides in length or even up to a few nucleotides, such as five or ten nucleotides, shorter than the full-length mRNA.

The polynucleotide, (i.e. the "antisense" polynucleotide), may be expressed in a cell from a suitable vector. A suitable vector is typically a recombinant replicable vector comprising a sequence which, when transcribed, gives rise to the polynucleotide (typically an RNA). Typically, the sequence encoding the polynucleotide is operably linked to a control sequence which is capable of providing for the transcription of the sequence giving rise to the polynucleotide. The term "operably linked" refers to a juxtaposition wherein the components described are in a

-19-

relationship permitting them to function in their intended manner. A control sequence "operably linked" to a sequence giving rise to an antisense RNA is ligated in such a way that transcription of the sequence is achieved under conditions compatible with the control sequences.

5 The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for transcription to occur and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for 10 example for the production of antisense RNA, or used to transfect or transform a host cell. The vector will typically be adapted for use *in vivo*, for example in a method of gene therapy.

Promoters/enhancers and other expression regulation signals may be selected 15 to be compatible with the host cell for which the expression vector is designed. For example, mammalian promoters, such as β -actin promoters, may be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the promoter rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters. All these promoters are 20 readily available in the art. Preferred promoters are tissue specific promoters, for example promoters driving expression specifically within vascular tissue.

In the antisense oligonucleotide approach, a suitable oligonucleotide will 25 typically have a sequence such that it will bind to the target mRNA. Therefore, it will typically have a sequence which has substantial complementarity to a part of such an mRNA. A suitable oligonucleotide will typically have substantial complementarity to a contiguous set of nucleotides within the target mRNA. An antisense oligonucleotide will generally be from about 6 to about 40 nucleotides in length. Preferably it will be from 12 to 20 nucleotides in length.

Generally the oligonucleotide used will have a sequence that is absolutely 30 complementary to the sequence. However, absolute complementarity may not be

-20-

required and in general any oligonucleotide having sufficient complementarity (i.e. substantial complementarity) to form a stable duplex (or triple helix as the case may be) with the target nucleic acid is considered to be suitable. The stability of a duplex (or triplex) will depend *inter alia* on the sequence and length of the hybridizing 5 oligonucleotide and the degree of complementarity between the antisense oligonucleotide and the target sequence. The system can tolerate less complementarity when longer oligonucleotides are used. However oligonucleotides, especially oligonucleotides of from 6 to 40 nucleotides in length, which have sufficient complementarity to form a duplex having a melting temperature of greater 10 than 40°C under physiological conditions are particularly suitable for use in the present invention. The polynucleotide may be a polynucleotide which hybridises to under conditions of medium to high stringency such as 0.03M sodium chloride and 0.03M sodium citrate at from about 50 to about 60 degrees centigrade.

Antisense oligonucleotides may be chemically modified. For example, 15 phosphorothioate oligonucleotides may be used. Other deoxynucleotide analogs include methylphosphonates, phosphoramidates, phosphorodithioates, N3'P5'- phosphoramidates and oligoribonucleotide phosphorothioates and their 2'-O-alkyl analogs and 2'-O-methylribonucleotide methylphosphonates.

Alternatively mixed backbone oligonucleotides (MBOs) may be used. MBOs 20 contain segments of phosphothioate oligodeoxynucleotides and appropriately placed segments of modified oligodeoxy- or oligoribonucleotides. MBOs have segments of phosphorothioate linkages and other segments of other modified oligonucleotides, such as methylphosphonate, which is non-ionic, and very resistant to nucleases or 2'- O-alkyloligonucleotides.

25 An nucleic acid suitable for use in the invention may act via an RNA interference (RNAi) mechanism. Such a nucleic acid is typically a double-stranded RNA and has a sequence substantially similar to part of the target mRNA. Preferred nucleic acids of this type are typically short, for example 15mers to 25mers, in particular 18mers to 23mers. These short nucleic acids may be referred to as 30 interfering RNAs (iRNAs).

The use of short nucleic acids of the type described above is preferred because such inhibitors do not appear to trigger viral defence mechanisms of higher organisms. Such nucleic acids can be used to inhibit translation of the mRNA.

Alternatively, small fragments of sequence encoding the target gene product 5 (or a sequence substantially similar thereto) may be provided, cloned back to back in a suitable vector. The vectors described above are suitable for expression of such back to back sequences. Expression of the sequence leads to production of the desired double-stranded RNA.

A nucleic acid suitable for use in a complex of the invention may comprise a 10 sequence which encodes an antigen. An antigen is a molecule which contains one or more epitopes that will stimulate a host's immune system to make a cellular antigen-specific immune response, or a humoral antibody response. A suitable nucleic acid sequence encoding an antigen can be derived from any known organism or pathogen, e.g. a virus, a bacterium, a parasite, a plant, a protozoan, or a fungus. The term also 15 includes tumor antigens. The antigen typically comprises one or more T cell epitopes. "T cell epitopes" are generally those features of a peptide structure capable of inducing a T cell response. In this regard, it is accepted in the art that T cell epitopes comprise linear peptide determinants that assume extended conformations within the peptide-binding cleft of MHC molecules, (Unanue *et al.* *Science* 236, 551- 20 557, 1987). As used herein, a T cell epitope is generally a peptide having about 8-15, preferably 5-10 or more amino acid residues. A nucleic acid suitable for use in a complex of the invention may encode such a T cell epitope.

The high levels of transfection make the complex of the invention particularly 25 suitable for the production of host cells capable of producing a desired protein, so-called "cell factories". Thus, a nucleic acid suitable for use in a complex of the invention may encode a protein that is commercially useful, for example industrially or scientifically useful, for example an enzyme; pharmaceutically useful, for example, a protein that can be used therapeutically or prophylactically as a medicament or vaccine; or diagnostically useful, for example, an antigen for use in an 30 ELISA.

A biologically-active material for use in a complex of the invention may be a peptide or polypeptide. Suitable peptides/polypeptides are those encoded by one of the nucleic acids set out above. Thus, the peptide/polypeptide may be, for example, one that is absent or deficient in a genetic disease or an antigen or immunogen.

5 Alternatively, the peptide/polypeptide may be, for example, a natural hormone such as tissue insulin, calcitonin and human growth hormone, or a synthetic analogue of such a natural hormone. Further peptides/polypeptides which may be used in a complex of the invention include interleukin-2, tumor necrosis factor, tissue plasminogen activator, factor VIII, erythropoietin, growth factors such as epidermal 10 growth factor, growth hormone releasing factor, neural growth factor and toxic peptides such as ricin, diphtheria toxin, or cobra venom factor, capable of eliminating diseased or malignant cells. Fragments of any of the polypeptides mentioned above may also be used in a complex of the invention.

Peptides/polypeptides suitable for use in a complex of the invention may be 15 chemically modified, e.g. post-translationally modified. For example, they may be glycosylated or comprise modified amino acid residues. They may also be modified by the addition of histidine residues to assist their purification or by the addition of a signal sequence to promote insertion into the cell membrane.

A biologically-active material for use in a complex of the invention may be a 20 small molecule. Preferred small molecules are therapeutic agents, for example steroids such as hydrocortisone, fluocinolone acetonide, fluocinonide and dexamethasone, non-steroidal anti-inflammatory agents such as 1-acetylsalicylic acid, antiviral nucleosides such as AZT, acyclovir and gancyclovir, or phospholipid derivatives of such antiviral nucleosides, antibiotics, anaesthetic agents, cytostatic 25 agent or immunomodulators. A complex of the invention may also contain a sunscreen or a cosmetic.

A complex of the invention will typically comprise a ratio of from 0.25 to 12: 1 by weight of a lipid of the invention: a biologically-active material (such as a nucleic acid), for example a ratio of from 0.5 to 8: 1 by weight of a lipid of the 30 invention: a biologically-active material, such as from 0.75 to 4: 1 weight of a lipid

of the invention: a biologically-active material, for example from 1 to 2: 1 by weight of a lipid of the invention: a biologically-active material.

A complex of the invention may comprise an integrin-binding component, for example an integrin-binding peptide. An integrin-binding component suitable for use in a complex of the invention is any such component which is capable of binding specifically to integrins found on the surface of cells. The integrin-binding component may be a naturally occurring integrin-binding ligand, for example, an extra-cellular matrix protein, a viral capsid protein, the bacterial protein invasin, a snake venom disintegrin protein, or an integrin-binding fragment of any such protein. Such integrin-binding proteins and fragments thereof may be obtained from natural sources or by recombinant techniques, but they are difficult to synthesise and purify in large amounts, they require conjugation directly to DNA or RNA or to polycationic elements for DNA or RNA binding, and are immunogenic *in vivo*.

It is thus preferable to use integrin-binding peptides, in particular because of their ease of synthesis, purification and storage, their potential for chemical modification, and their potentially low immunogenicity *in vivo*. Examples of integrin-binding peptides are given in Verfaillie, 1994 #635; Wang, 1995 #645; Staatz, 1991 #539; Pierschbacher, 1984 #314; Massia, 1992 #86; Clements *et al.* J. Cell Science 107, 2127-2135, 1994; Lu *et al.*, Biochemistry J. 296, 21-24, 1993; and in Koivunen *et al.*, Biol/Technology 13, 265-270, 1995; Koivunen *et al.*, Biological Chemistry 268, 20205-20210, 1993; Koivunen *et al.*, J. Cell. Biology 124(3), 373-380, 1994; O'Neil *et al.*, Proteins 14, 509-515, 1992; Healy *et al.*, Biochemistry 34, 3948-3955, 1995; and Pasqualani *et al.*, J. Cell. Biology 130, 1189-1196, 1995.

As indicated above, peptides containing the conserved amino acid sequence arginine-glycine-aspartic acid (RGD) bind with high affinity to integrins. Accordingly, peptides comprising the RGD sequence are particularly useful. The affinity between integrin and peptide ligands is influenced by the amino acid sequence flanking the RGD domain. Peptides having a cyclic region in which the conformational freedom of the RGD sequence is restricted generally have a higher affinity for integrin receptors than do their linear counterparts. Such cyclic peptides

are particularly preferred. Cyclic peptides may be formed by the provision of two cysteine residues in the peptide, thus enabling the formation of a disulphide bond. A cysteine residue may be separated from the RGD sequence by one or more residues, for example, up to six residues, or may be immediately adjacent to the RGD sequence, although preferably both cysteines are not immediately adjacent to the ends of the RGD sequence.

An example of an amino acid sequence that will permit cyclisation by disulphide bond formation is CRGDMFGC. A peptide that consists of or comprises the sequence CRGDMFGC may advantageously be used as an integrin-binding peptide according to the present invention. Examples of peptides that comprises the sequence CRGDMFGC and that are effective integrin-binding ligands are the peptides GGCRGDMFGC, GGCRGDMFGCG, GGCRGDMFGCA and GACRGDMFGCA.

The peptide GACDCRGDCFCA has the potential to form two disulphide bonds for stabilising the RGD loop. That peptide and others having the potential to form two RGD-stabilising disulphide bonds, may be particularly useful as integrin-binding ligands according to the present invention.

A further useful peptide is GACATRWARECG.

However, not all integrin-binding peptides contain the conserved RGD sequence. For example, the peptides GACRRETAWACA, GACRRETAWACG and XSXGACRRETAWACG are integrin-specific peptides. Other peptides comprising the sequence CRRETTAWAC or CRRETAWAC may be used, as may other non-RGD peptides, particularly those that have the potential for disulphide bond formation.

Peptide sequences may be designed on the basis of known ligands, for example, on the basis of integrin-binding domains of naturally-occurring integrin-binding ligands, or on the basis of known peptides that bind to integrins.

As stated above, integrins are a family of heterodimeric proteins found on the surface of cells. They consist of several different α and β subunits. Some integrins are found on many types of cells, others are more specific, for example, $\alpha 5$ and αv

-25-

integrins are widespread and are found on a diverse range of cells. Integrin-binding ligands can vary in their affinity for different integrins. For example, GACRGDMFGCA (peptide 1) has affinity for $\alpha 5$ and αv integrins but is non-specific (O'Neil *et al.* 1992, *supra*; Hart *et al.* 1997, *supra*). GACDCRGDCFCA (peptide 5) has high affinity for integrin αv but is not αv -specific (Koivunen *et al.* 1995, *supra*; Hart *et al.* 1997, *supra*). GACRRETAWACG, however, which does not contain the conserved RGD region, is $\alpha 5\beta 1$ -specific (Koivunen *et al.* 1995, *supra*). Various integrin-binding peptides and their integrin specificity are set out below:

10	<u>Peptide number and integron specificity</u>	<u>Sequence</u>
	Peptide 1 (αv , $\alpha 5\beta 1$)	GACRGDMFGCA
	Peptide 2 (αv , $\alpha 5\beta 1$)	GACRGDMFGCGG
15	Peptide 5 (αv)	GACDCRGDCFCA
	Peptide 6 ($\alpha 5\beta 1$)	GACRRETAWACG
	Peptide 7 ($\alpha 4\beta 1$)	GAGPEILDVPST
	Peptide 8 ($\alpha 4\beta 1$)	GACQIDSPCA
	Peptide 9 ($\alpha 5\beta 1$)	GACRRETAWACGKGACRRETAWACG

20

Yet further possibilities include GA-CXCG where X is SERSMNF, YGLPHKF, PSGAARA, VKSMVTH or LQHKSMP.

25 Alternative oligopeptides may be used in conjunction with or instead of an integrin-binding peptide, for example alternative targetting ligands, such as oligopeptides identified by panning with phage-based peptide-display libraries; membrane-active peptides such as melittin; fragments of the HIV tat protein; single chain Fv regions of antibodies; VP22; peptides containing nuclear localisation sequences; mitochondrial localisation sequences; and peptides based on the influenza virus haemagglutinin protein.

30

A complex of the invention will typically comprise a ratio of from 0.25 to 4:

4 by weight of a lipid of the invention: an integrin-binding peptide, for example a ratio of from 0.5 to 2: 4 by weight of a lipid of the invention: an integrin-binding peptide, such as from 0.75 to 1: 4 by weight of a lipid of the invention: an integrin-binding peptide.

5 In general, complexes of the invention will comprise a cationic component, such as a cationic polymer. This is particularly the case for complexes where the biologically-active material is a nucleic acid. Cationic polymers suitable for use in the invention are typically capable of binding to a nucleic acid. Especially preferred cationic polymers are capable of condensing nucleic acids into a particle with a
10 diameter of from about 50nm to about 150nm. Generally, suitable cationic polymers are low molecular weight polymers and the number of positive charges per polymer molecule is typically from about 7 to about 50, preferably from about 7 to about 25 or more preferably from about 12 to about 16. Suitable cationic polymers may have any number of cationic monomers, although generally the polymer must retain the ability
15 to bind nucleic acids. For example, from 3 to 100 cationic monomers may be present, for example, from 10 to 20.

20 Suitable polymers include oligolysine, for example having from 10 to 20 lysine residues, for example, from 15 to 17 residues, especially 16 residues, i.e. $[K]_{16}$. Thus, poly-L-lysine (pLL) which has a molecular weight of 3.4kDa (and which has an average of 16 positive charges per molecule) is preferred. Other suitable polymers include polyethylenimine (PEI) which has a molecular weight of 2kDa (with an average of 12 positive charges per molecule at neutral pH) and polyamidoamine dendrimers. Suitable peptides are disclosed in USSN 09/424656 and 08/836786, the disclosures of which are incorporated herein by reference.

25 The polymers pLL and pEI will condense RNA and DNA in water or 10mM HEPES. Typically, cationic polymers with a pKa of greater than 9.0 (e.g. pLL) generally do not possess endosomolytic activity and require the presence of an endosome-disrupting agent, for example chloroquine, to enable them to gain access into the cytosol.

30 The polycationic component may advantageously be linked or otherwise

-27-

attached to the integrin-binding component. For example, a polycationic polymer may be chemically bonded to an integrin-binding component, for example, by a peptide bond in the case of an oligolysine. Other types of suitable bonds are thioether and disulphide bonds. The polycationic component may be linked at any 5 position of the integrin-binding component. Preferred combinations of integrin-binding component and polycationic polymer are an oligolysine, especially $[K]_{16}$, linked via a peptide bond to an integrin-binding peptide peptide, for example any one of the peptides set out described above.

A complex of the invention will typically comprise a ratio of from 0.25 to 4: 10 4 by weight of a lipid of the invention: a polycationic component (such a polycationic peptide), for example a ratio of from 0.5 to 2: 4 by weight of a lipid of the invention: a polycationic component, such as from 0.75 to 1: 4 by weight of a lipid of the invention: a polycationic component.

Where the integrin-binding peptide and polycationic component are combined 15 the ratio of a lipid of the invention to the combined peptide is as given above for an integrin-binding peptide and polycationic component individually.

Agents other than peptides may also be introduced onto the condensing 20 cationic polymer, for example saccharide residues or lipids, to modulate solubility and interaction of formulations with biological proteins, fluids, membranes and cells, including to specific receptors.

A neutral lipid may be used in a complex of the invention. However, a complex of the invention may be free of a neutral lipid. Any neutral lipid may be used in a complex of the invention, although typically those that have membrane 25 destabilising properties are preferred. An example of a suitable neutral lipid which has membrane destabilising properties is dioleyl phosphatidylethanolamine (DOPE). DOPE has membrane destabilising properties sometimes referred to as "fusogenic" properties. The ratio of neutral lipid: a lipid of the invention is from about 0.5 to 2: 1 for example 1: 1.

The total amount of lipid in comparison to the other components of a 30 complex of the invention is as set out above for a lipid of the invention individually.

-28-

A complex of the invention may be prepared by a process which comprises admixing the components of the complex. Although, the components may be admixed in any order, it is generally preferable that the lipid component is not added last. In the case where there is a combined integrin-binding peptide/polycationic component, it is generally preferable to combine the components in the following order: lipid; combined integrin-binding peptide/polycationic component; biologically-active material. The components of a complex of the invention are preferably admixed in the amounts set out above.

Thus a typical complex of the invention may be prepared by admixing a lipid of the invention/combined integrin-binding peptide-cationic component (such as $[K]_{16}$ GACRRETAWACG)/nucleic acid (such as DNA) in the weight ratio 0.75:4:1, 1:4:1 or 2:4:1. If a neutral lipid is present, it is typically present in a weight ratio of 1:1 lipid of the invention: neutral lipid and the total amount of lipid relative to the other components is as set out in the previous sentence.

The invention also provides a mixture which comprises: a lipid; and one or more of an integrin-binding peptide, a polycationic component and a neutral lipid. All of the components of such a mixture may be as set out above. Such a mixture may be used to produce a biologically-active material containing complex of the invention by the incorporation of a biologically-active material with the mixture, for example by admixture.

The present invention further provides a process for the production of a complex of the present invention, which comprises admixing a biologically-active material with a mixture of the invention. The preferred components, preferred combinations of components, preferred ratios of components and preferred order of mixing, both with regard to the mixture and to the production of a complex are as described above in relation to the complex of the invention.

A complex of the invention may be used in a process for transfecting a cell with a biologically-active material. In such a process, a host cell is contacted with a complex of the invention. A complex of the invention may also be used in a process for expressing a nucleic acid in a host cell. Such a process comprises contacting the

-29-

host cell with a complex of the invention which comprises a nucleic acid. The host cell is then subjected to conditions that enable the cell to express the nucleic acid, for example it is cultured in a medium which allows expression of the nucleic acid.

5 A complex of the invention may be further used in a process for the production of a polypeptide. In such a method a host cell is transfected with a nucleic acid using the method set out above. The transfection is carried out under conditions suitable for expression of the polypeptide encoded by the nucleic acid or, alternatively, the transfected cell is transferred to conditions suitable for expression of the polypeptide encoded by the nucleic acid. The polypeptide may then be 10 recovered from the host cell or from the culture medium.

In all of the methods set out above, the host cell may be any host cell. Thus, the cell may be a prokaryotic cell or a eukaryotic cell. The cell may be from a bacterium, a mycobacterium, a protozoan, a parasite, a fungus a plant or an animal. Suitable animal cells are mammalian cells, for example human cells.

15 All of the methods may be carried out *in vivo*, *in vitro* or *ex vivo*. Also, cells may be obtained from a host, transfected according to the method set out above, and then returned to the host. The invention also provides a cell transfected with a complex of the invention and progeny cells derived from such a transfected cell.

20 The various components that make up the complex of the invention may be pre-mixed or they may be packaged, for example in a two or more part kit, for mixing shortly prior to or during administration. Thus, the invention also provides a kit for delivery of a biologically-active material to a cell, which kit comprises a mixture of the invention or components suitable for the preparation of a mixture of the invention. Thus, a kit may comprise a lipid of formula (I) or (II) as set out above 25 and one or more of an integrin-binding peptide, a polycationic component and a neutral lipid (all as described above). The kit may also comprise a biologically-active material. For example, the kit may comprise a nucleic acid, optionally in the form of a plasmid or vector which may be empty or comprise a coding sequence.

30 A kit of the invention may comprise appropriate buffers and/or control cells. Also, a kit of the invention may comprise appropriate packaging and instructions for

-30-

using the kit in one of the methods set out above. Thus, the instructions may indicate the preferred ratios of the components and the preferred order of admixing the components, for example as described above. A kit may be used for producing a complex suitable for use in gene therapy, vaccination, antisense or iRNA therapy.

5 Alternatively, it may be used for producing a complex suitable for transfecting a host cell with a nucleic acid encoding a commercially useful protein, i.e. to produce a so-called "cell" factory.

A complex of the invention may be used in a method of treatment or vaccination. Treatment according to the invention may be prophylactic or

10 therapeutic.

Typically, treatment according to the invention is by gene therapy, anti-sense therapy or iRNA therapy. Thus, a complex of the invention may be used in a method for nucleic acid transfer, for example in a method of treatment of the human or animal body by therapy. A complex of the invention may also be used for the

15 manufacture of a medicament for use in nucleic acid transfer, for example in treatment of the human or animal body by therapy, especially in the treatment of a condition caused by or related to a genetic defect or modification. The condition of a patient suffering from such a condition can be improved by administration of a complex of the invention. A therapeutically effective amount of a complex of the

20 invention may be given to a host in need thereof. The host may be a human or non-human animal.

Targets for gene therapy are well known and include monogenic disorders, for example, cystic fibrosis, various cancers, and infections, for example, viral infections, for example, with HIV. For example, transfection with the p53 gene offers great potential for cancer treatment. Targets for gene vaccination are also well known, and include vaccination against pathogens for which vaccines derived from natural sources are too dangerous for human use and recombinant vaccines are not always effective, for example, hepatitis B virus, HIV, HCV and herpes simplex virus. Targets for anti-sense therapy are also known. Further targets for gene therapy and anti-sense therapy are being proposed as knowledge of the genetic basis of disease

25

30

-31-

increases, as are further targets for gene vaccination.

A complex of the invention may be used in vaccination. Thus, a complex of the invention may be used to deliver an antigen or a nucleic acid encoding an antigen. That is to say, a complex of the invention may be used in gene vaccination. A complex of the invention may be used to elicit an immune response against a wide variety of antigens for the treatment and/or prevention of a number of conditions including, but not limited to, cancer, allergies, toxicity and infection by pathogens such as viruses, bacteria, fungi, and other pathogenic organisms.

Suitable viral antigens and nucleic acids encoding such antigens for use in the complexes of the invention include, but are not limited to, those obtained or derived from the hepatitis family of viruses, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), the delta hepatitis virus (HDV), hepatitis E virus (HEV) and hepatitis G virus (HGV). See, e. g., International Publication Nos. WO 89/04669; WO 90/11089; and WO 90/14436. The HCV genome encodes several viral proteins, including E1 and E2. See, e. g., Houghton et al. (1991) Hepatology 14 : 381-388. Nucleic acid molecules containing sequences encoding these proteins, as well as antigenic fragments thereof, will find use in the present methods. Similarly, the coding sequence for the 8-antigen from HDV is known (see, e. g., U. S. Patent No. 5,378,814).

In like manner, a wide variety of proteins, and nucleic acids encoding such proteins, from the herpesvirus family can be used as antigens in the present invention, including proteins derived from herpes simplex virus (HSV) types 1 and 2, such as HSV-1 and HSV-2 glycoproteins gB, gD and gH; antigens from varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) including CMV gB and gH; and antigens from other human herpesviruses such as HHV6 and HHV7. (See, e. g. Chee et al. (1990) Cytomegaloviruses (J. K. McDougall, ed., Springer Verlag, pp. 125-169; McGeoch et al. (1988) J. Gen. Virol. 69: 1531-1574; U. S. Patent No. 5,171,568; Baer et al. (1984) Nature 310: 207-211; and Davison et al. (1986) J. Gen. Virol. 67: 1759-1816.)

Human immunodeficiency virus (HIV) antigens, such as gpl20 molecules for

5 a multitude of HIV-1 and HIV-2 isolates, including members of the various genetic subtypes of HIV, are known and reported (see, e. g., Myers et al., Los Alamos Database, Los Alamos National Laboratory, Los Alamos, New Mexico (1992); and Modrow et al. (1987) J. Virol. 61: 570-578) and antigen-containing nucleic acid sequences derived or obtained from any of these isolates will find use in the present invention.

10 Furthermore, other immunogenic proteins derived or obtained from any of the various HIV isolates will find use herein, including nucleic acid sequences encoding one or more of the various envelope proteins such as gap 160 and gp41, gag antigens such as p24gag and p55gag, as well as proteins derived from the pol, env, tat, vif, rev, nef, vpr, vpu and LTR regions of HIV.

15 Antigens derived or obtained from other viruses will also find use herein, such as without limitation, antigens from members of the families Picornaviridae (e. g., polioviruses, rhinoviruses, etc.); Caliciviridae; Togaviridae (e. g., rubella virus, dengue virus, etc.); Flaviviridae; Coronaviridae; Reoviridae (e. g., rotavirus, etc.); Birnaviridae ; Rhabdoviridae (e. g., rabies virus, etc.); Orthomyxoviridae (e. g., influenza virus types A, B and C, etc.); Filoviridae; Paramyxoviridae (e. g., mumps virus, measles virus, respiratory syncytial virus, parainfluenza virus, etc.); Bunyaviridae; Arenaviridae; Retroviridae (e. g., HTLV-I ; HTLV-11 ; HIV-1 (also known as HTLV-111, LAV, ARV, hTLR, etc.)), including but not limited to antigens from the isolates HIVIIB, HIVSF2, HTVLAV, HIVLAI, HIVMN) ; HIV-1CM235, HIV-1 ; HIV-2, among others; simian immunodeficiency virus (SIV); Papillomavirus, the tick-bourne encephalitis viruses; and the like. See, e. g. Virology, 3rd Edition (W. K. Joklik ed. 1988); Fundamental Virology, 2nd Edition (B. N. Fields and D. M. Knipe, eds. 1991), for a description of these and other viruses. Nucleic acid sequences encoding such antigens may of course also be used in a complex of the invention.

30 In some contexts, it may be preferable that a selected antigen is obtained or derived from a viral pathogen that typically enters the body via a mucosal surface and is known to cause or is associated with human disease, such as, but not limited to,

-33-

HIV (AIDS), influenza viruses (Flu), herpes simplex viruses (genital infection, cold sores, STDs), rotaviruses (diarrhea), parainfluenza viruses (respiratory infections), poliovirus (poliomyelitis), respiratory syncytial virus (respiratory infections), measles and mumps viruses (measles, mumps), rubella virus (rubella), and rhinoviruses (common cold). Again, nucleic acid sequences encoding such antigens may be used in a complex of the invention.

Suitable bacterial and parasitic antigens can be obtained or derived from known causative agents responsible for diseases including, but not limited to, Diphteria, Pertussis, Tetanus, Tuberculosis, Bacterial or Fungal Pneumonia, Otitis 10 Media, Gonorrhea, Cholera, Typhoid, Meningitis, Mononucleosis, Plague, Shigellosis or Salmonellosis, Legionaire's Disease, Lyme Disease, Leprosy, Malaria, Hookworm, Onchocerciasis, Schistosomiasis, Trypanosomiasis, Lesmaniasis, Giardia, Amoebiasis, Filariasis, Borelia, and Trichinosis. Still further antigens can be obtained or derived from unconventional pathogens such as the causative agents of 15 kuru, Creutzfeldt-Jakob disease (CJD), scrapie, transmissible mink encephalopathy, and chronic wasting diseases, or from proteinaceous infectious particles such as prions that are associated with mad cow disease. Again, nucleic acid sequences encoding such antigens may be used in a complex of the invention.

Specific pathogens from which antigens can be derived include M. tuberculosis, Chlamydia, N. gonorrhoeae, Shigella, Salmonella, Vibrio Cholera, 20 Treponema pallidua, Pseudomonas, Bordetella pertussis, Brucella, Francisella tulorensis, Helicobacter pylori, Leptospria interrogans, Legionella pneumophila, Yersinia pestis, Streptococcus (types A and B), Pneumococcus, Meningococcus, Hemophilus influenza (type b), Toxoplasma gondii, Complylebacteriosis, 25 Moraxella catarrhalis, Donovanosis, and Actinomycosis ; fungal pathogens including Candidiasis and Aspergillosis; parasitic pathogens including Taenia, Flukes, Roundworms, Amebiasis, Giardiasis, Cryptosporidium, Schistosoma, Pneumocystis carinii, Trichomoniasis and Trichinosis. Thus, the present invention can also be used to provide a suitable immune response against numerous veterinary diseases, such as 30 Foot and Mouth diseases, Coronavirus, Pasteurella multocida, Helicobacter,

-34-

Strongylus vulgaris, Actinobacillus pleuropneumonia, Bovine viral diarrhea virus (BVDV), Klebsiella pneumoniae, E. coli, Bordetella pertussis, Bordetella parapertussis and brochiseptica. Again, nucleic acid sequences encoding an antigen as set out above may be used in a complex of the invention.

5 Typically, a nucleotide sequence corresponding to (encoding) one or more of the above-listed antigen(s) is used in a complex of the invention.

A complex of the invention may be in the form of a pharmaceutical composition which additionally comprises a pharmaceutically-acceptable carrier, diluent or excipient for example water or a physiologically-acceptable buffer.

10 Similarly, a vaccine composition comprises a complex of the invention and a pharmaceutically-acceptable carrier, diluent or excipient for example water or a physiologically-acceptable buffer.

Such pharmaceutical and vaccine compositions may be supplied in any suitable dispenser, for example a puffer.

15 A complex of the invention may be administered in a variety of dosage forms. Thus, it can be administered orally, for example as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules. The complexes may also be administered parenterally, either subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques. The complexes may also be 20 administered as suppositories. A physician will be able to determine the required route of administration for each particular patient.

The formulation of a complex for use in prophylaxis, treatment or vaccination will depend upon factors such as the nature of the exact complex, whether a pharmaceutical or veterinary use is intended, etc. A complex of the invention may be 25 formulated for simultaneous, separate or sequential use.

A complex of the invention is typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For example, solid oral forms may contain, together with the active compound, diluents, 30 e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants,

-35-

e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, gum arabic, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate; effervescent mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tabletting, sugar-coating, or film-coating processes.

10 Liquid dispersions for oral administration may be syrups, emulsions or suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol.

15 Suspensions and emulsions may contain as carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may contain, together with the active compound, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired, a suitable amount of lidocaine hydrochloride.

20 Solutions for intravenous administration or infusion may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

25 A therapeutically effective amount of a complex is administered to a patient. The dose of a complex may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular patient.

30 Typically, the amount of nucleic acid delivered in a complex of the invention will be in the range of from 1 μ g to 1g, preferably from 100 μ g to 10mg, according to the activity of the specific formulation, the age, weight and conditions of the subject

-36-

to be treated, the type and severity of the degeneration and the frequency and route of administration. A single dose may be administered daily or alternatively, may multiple doses, for example two, three, four or five doses may be administered daily.

5 The amount of nucleic acid referred to above may represent the total amount administered in the treatment regime or may represent each separate administration in the regime.

10 If the complex is to be used in vaccination, it may be administered to the host in one or more administrations. Typically after the initial administration a "booster" can be given. Typically the host is given 1, 2, 3 or more separate administrations, each of which is separated by at least 12 hours, 1 day, 2 days, 7 days, 14 days, 1 month or more.

15 The invention is further illustrated by the following reaction schemes and specific examples.

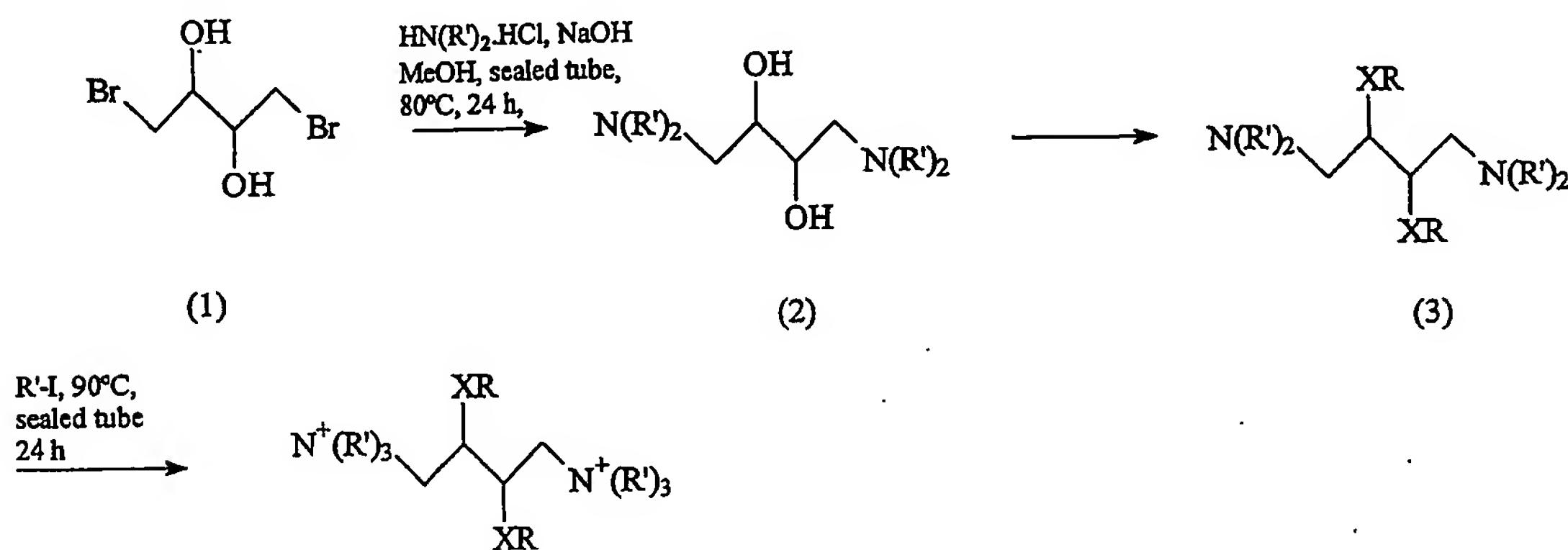
20

Reaction Schemes

The lipids of the invention can be prepared by analogy with known methods.

For example, compounds of formula (I) can be prepared from 1,4-dibromobutanediol, using dimethylamine in methanol, according to the following reaction scheme.

5



R' in the above reaction scheme is, of course, defined as R^3 and R^4 above. X in the above reaction scheme is defined as X^1 and X^2 above. R in the above reaction scheme is defined as R^1 and R^2 above.

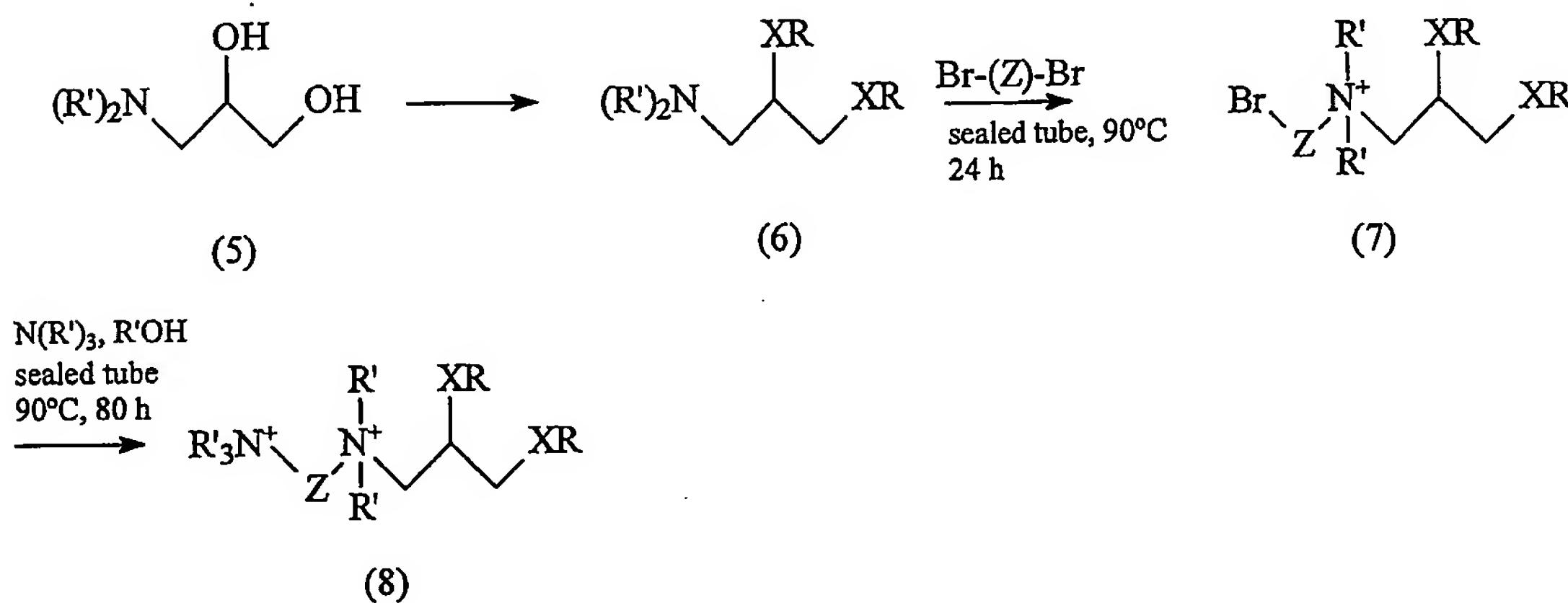
Each R' in the above reaction scheme can be the same or different. When the 10 two $\text{N}(\text{R}')_2$ moieties in the formula (2) are different, step (1) is typically conducted stepwise. A first $-\text{N}(\text{R}')_2$ moiety is added in a first step and a second $-\text{N}(\text{R}')_2$ moiety is added in a second step. If necessary, one of the hydroxy groups on compound (1) can be protected by a protecting group, prior to such a two stage reaction. The protecting group would, of course, then be removed after introduction of the first 15 $\text{N}(\text{R}')_2$ moiety and before the introduction of the second $\text{N}(\text{R}')_2$ moiety.

When X is $-\text{O}-\text{CH}_2-$, step 2 (introduction of the XR moieties) can be effected by reaction with $\text{L}-\text{R}$, wherein L is a leaving group such as a mesylate, in the presence of NaH in THF under reflux. When X is $-\text{O}-\text{CO}-$, the XR moieties can be introduced by reaction with RCO_2H , in the presence of EDCl , DMAP , triethylamine and DCM . Typically, the reaction is effected in a dark environment at room 20 temperature.

When the two XR moieties are different, typically, one XR moiety is introduced in a first step and a second XR moiety is introduced in a second step. One of the hydroxy groups on compound (2) can, if necessary, be protected prior to such a two stage reaction step by a standard hydroxy protecting group. The protecting group would of course then be removed after introduction of the first XR moiety and before introduction of the second XR moiety.

5 Although an iodine anion is used in the above scheme it will be appreciated that any suitable anion could be used.

10 Compounds of formula (II) in which R^6 is $-[A-Y]_n-R^4$ can be prepared as follows.



15 R' in the above reaction scheme is, of course, defined as R^3 and R^4 above. X in the above reaction scheme is defined as X^1 and X^2 above. R in the above reaction scheme is defined as R^1 and R^2 above. Z in the above reaction scheme corresponds to the moiety $-[A-Y]_nR^4$ except for the absence of the terminal $-N^+(R^4)_3$.

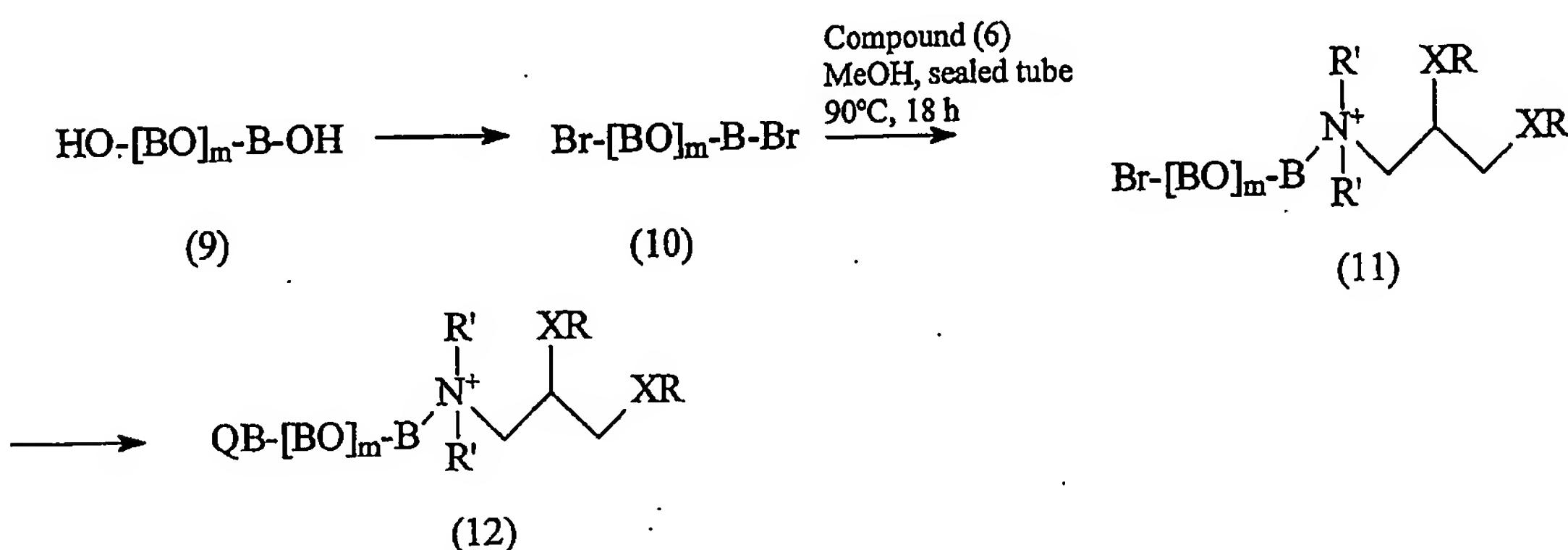
Introduction of the XR moieties in the above reaction scheme (step 1) can be effected as previously described.

20 If necessary, the Z group can comprise carbamate protected amino moieties in place of quaternary ammonium moieties. Under such circumstances, the carbamate

-39-

protected amino moieties in compound (8) can be deprotected and converted to quaternary ammonium moieties by standard methods. Quaternisation can, for example, be effected by reaction with R⁴-I. The compounds (8) are generally purified by recrystallisation.

5 Compounds of formula (II) in which R⁶ is -[BO]_m-Q can be prepared according to the following reaction scheme.



10 Introduction of the moiety Q can be effected by standard techniques. For example, when Q is -N⁺(R³)₃, it can be introduced by reacting compound (11) with a corresponding amino compound. Thus, for example, the moiety N⁺(CH₃)₃ can be introduced by reaction with trimethylamine (45 wt% in H₂O) in the presence of methanol in a sealed tube at 90°C for 24 hours.

15 When Q is a halogen other than bromine, it may, of course, be convenient to conduct the synthesis using a compound (10) which is appropriately halogenated. Similarly, when Q is OH, appropriate compounds (12) can be prepared by reacting a compound (9) with HBr (48% in H₂O), in toluene under reflux for 72 hours, to prepare a compound HO-[BO]_m-B-Br. This can, of course, be used in the above synthesis in place of compound (10) to yield compounds (12) in which Q is hydroxy.

20 Compounds in which Q is OR' and OC(O)R' can be prepared from corresponding compounds in which Q is hydroxy by known techniques.

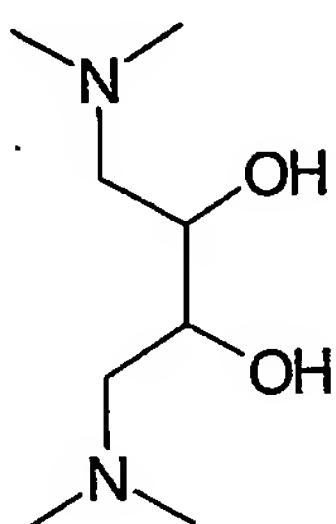
-40-

EXAMPLESExample 1

In Example 1 Reaction Scheme I was followed to produce

5 1,4-Di(trimethylammonium)-2,3-dioleoyloxy-butane; diiodide.

The first step was the production of 1,4-Di(dimethylamino)-2,3-butanediol



10

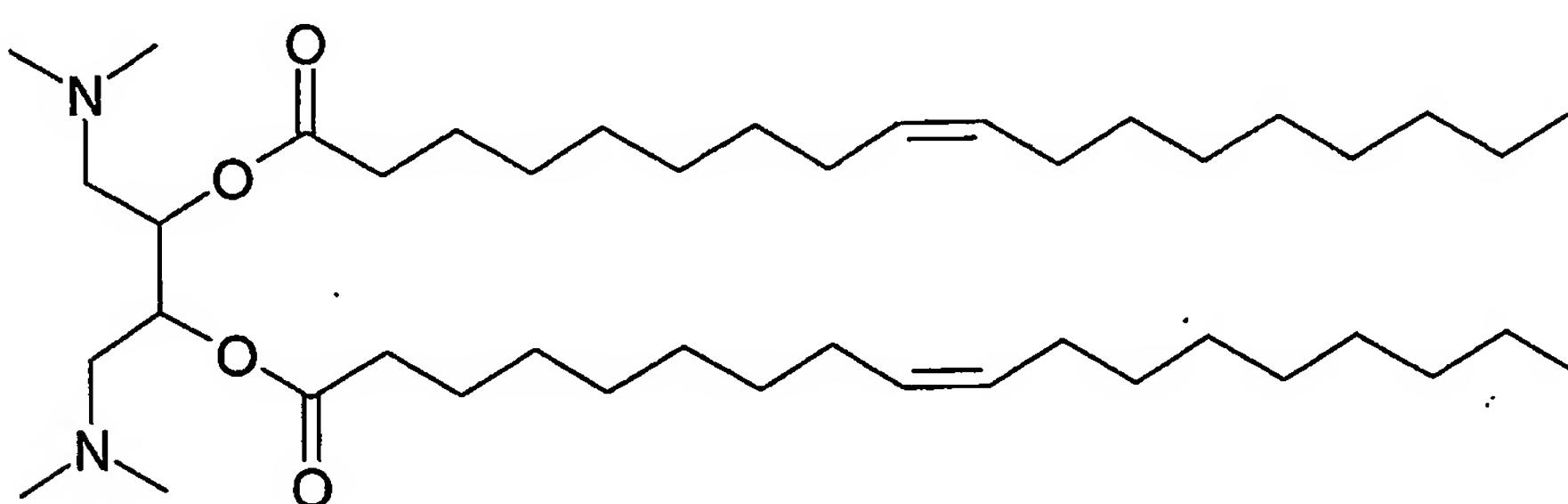
Powdered sodium hydroxide (1.28 g, 32.0 mmol) was stirred in methanol (7 ml) at 0 °C. Dimethylamine hydrochloride (1.96 g, 24.0 mmol) was added followed by 1,4-Dibromo-2,3-butanediol (1.00 g, 4.00 mmol). The mixture was then heated to 80 °C in a sealed tube for 24 hr. The mixture was then concentrated *in vacuo*. The residue was re-dissolved in chloroform (25 ml) and the resulting mixture was filtered. The filtrate was concentrated *in vacuo* to yield the titled product as clear oil which solidifies to a colourless waxy solid upon refrigeration (0.69 g, 98%).

15 δ_H (300 MHz, CDCl₃) 2.27 (12H, s, NCH₃), 2.35 (2H, dd, *J* 12.6 Hz and *J* 4.9 Hz, NCH₂), 2.60 (2H, dd, *J* 12.5 Hz and *J* 8.2 Hz, NCH₂), 3.65 (2H, m, CHOH), 4.45 (2H, brs, OH);
 δ_C (75 MHz, CDCl₃) 46.23 (NCH₃), 62.68 (NCH₂), 69.23 (CHOH);
m/z (APCI+) 177.2 (90%, M+1), 132.2 (100%);

ν_{max} cm⁻¹ (Film) 3417.6, 2920.0, 2783.1, 2343.4, 1633.6, 1461.9;

The 1,4-di(dimethylamino)-2,3-butane diol prepared above was then used to synthesise 1,4-Di(dimethylamino)-2,3-dioleoyloxy-butane.

5



1,4-Di(dimethylamino)-2,3-butanediol (0.30 g, 1.70 mmol), EDCI (0.98 g, 5.1 mmol) and triethylamine (1.42 ml, 10.2 mmol) were stirred in anhydrous DCM (35 ml) at r.t. Oleic acid (1.44 g, 5.10 mmol) and DMAP (62.0 mg, 30 mol %) were and stirring was continued in the dark at r.t. Water (50 ml) was added and the mixture was separated and the chlorinated layer was subsequently washed with saturated sodium hydrogencarbonate solution (50 ml) and brine (50 ml). The chlorinated layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The product was purified by flash chromatography (gradient; DCM to 10% methanol in DCM) to obtain the above product as a clear yellow oil which solidified upon refrigeration (0.59 g, 50%).

R_f = 0.80 (20% methanol in DCM);

δ_{H} (300 MHz, CDCl₃) 0.85 (6H, t, *J* 6.93 Hz, CH₂CH₃), 1.25 (40H, m), 1.60 (4H, m, O₂CCH₂CH₂), 2.00 (8H, m, CH₂CH=CHCH₂), 2.24 (12H, s, NCH₃), 2.32 (8H, m, NCH₂ and O₂CCH₂CH₂), 5.22 (2H, m, CHO₂C), 5.35 (4H, m, CH=CH); δ_{C} (75 MHz, CDCl₃) 14.44 (CH₂CH₃), 23.04 – 32.27, 34.74 (O₂CCH₂CH₂), 46.24

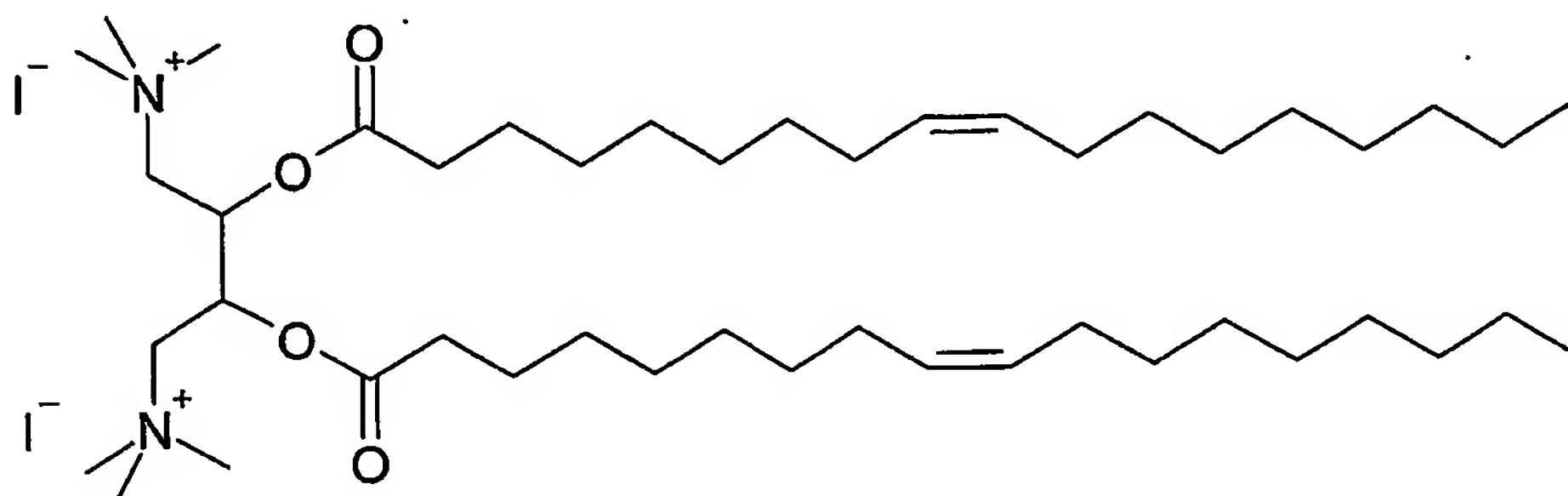
-42-

(NCH₃), 59.96 (NCH₂), 70.48 (CHO₂CCH₂), 130.07 and 130.37 (CH=CH), 173.43 (C=O, ester);

m/z (APCI+) 705.8 (45%, M+1), 438.6 (100%);

ν_{max} cm⁻¹ (Film) 3408.0, 2923.9, 2854.5, 2769.6, 2337.6, 1739.7, 1461.9;

5 In a third step 1,4-Di(trimethylammonium)-2,3-dioleoyloxy-butane; diiodide was formed



10 1,4-Di(dimethylamino)-2,3-dioleoyloxy-butane (296 mg, 0.42 mmol) and iodomethane (2.00 ml) in methanol (2 ml) were stirred in a sealed tube at 90 °C for 18 hr. The solvents were removed *in vacuo* to yield a residue. Ethyl acetate was added to the residue and an insoluble yellow solid was collected by filtration to yield the above product as a yellow waxy solid (385 mg, 93%).

15 δ_{H} (300 MHz, CDCl₃) 0.85 (6H, t, *J* 6.9 Hz, CH₂CH₃), 1.25 (40H, m), 1.60 (4H, m, O₂CCH₂CH₂), 2.00 (8H, m, CH₂CH=CHCH₂), 2.32 (4H, m, O₂CCH₂CH₂), 3.64 (18H, brs, NCH₃), 4.30 (2H, brs, NCH₂), 4.70 (2H, brs, NCH₂), 5.35 (4H, brm, CH=CH), 5.71 (2H, brm, CHO₂C);

20 δ_{C} (75 MHz, CDCl₃) 14.48 (CH₂CH₃), 23.04 – 32.27, 34.79 (O₂CCH₂CH₂), 55.86 (NCH₃), 66.85 (NCH₂), 77.62 (CHO₂CCH₂), 129.98 and 130.47 (CH=CH), 172.99 (C=O, ester);

-43-

m/z (ESP+) 367.6 (100%, $\frac{1}{2}M^+$);

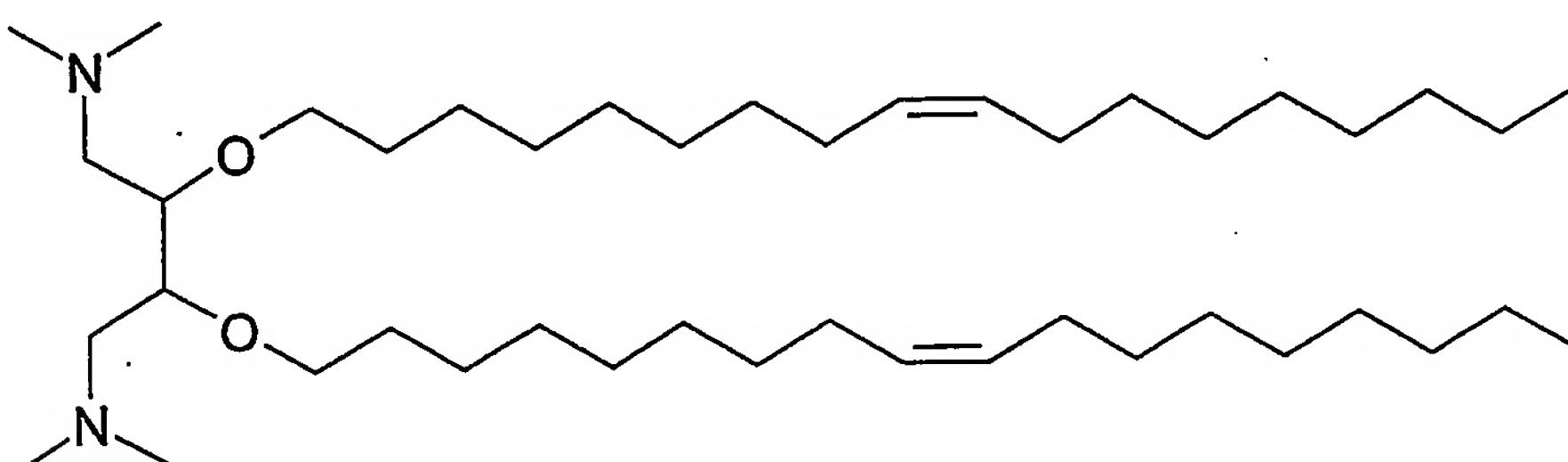
ν_{max} cm^{-1} (nujol mull) 3399.8, 2916.6, 2854.0, 2708.4, 2353.9, 1745.4, 1460.0;

Example 2

5

Example 2 also illustrates Reaction Scheme 1. The first step of Example 1 was repeated, but in the second step the resulting butane diol was then used to synthesise an ether, rather than ester based lipid, namely 1,4-Di(dimethylamino)-2,3-dioleyloxybutane.

10



1,4-Di-(dimethylamino)-2,3-butanediol (0.62 g, 3.50 mmol) was added to a stirred solution of sodium hydride (60%, 0.40 g, 10.5 mmol) in anhydrous THF (40 ml).

15

The mixture was heated at reflux for 4 hr. Oleyl mesylate (3.64 g, 10.5 mmol) was added and the resulting mixture was heated at reflux for 48 hr. Water (50 ml) was added and the mixture was extracted with ethyl acetate (3 x 50 ml). The combined organic extracts were washed with saturated sodium hydrogencarbonate solution (50 ml) and brine (50 ml) and dried over anhydrous magnesium sulfate. The solvents were removed *in vacuo*. The product was purified by flash chromatography (gradient; DCM to 20% methanol in DCM) to obtain the titled product as an orange oil (0.40 g, 17%).

-44-

R_f = 0.30 (10% methanol in DCM);

δ_H (300 MHz, $CDCl_3$) 0.91 (6H, t, J 6.93 Hz, CH_2CH_3), 1.25 (44H, m), 1.55 (4H, m, OCH_2CH_2), 2.00 (8H, m, $CH_2CH=CHCH_2$), 2.24 (14H, s + dd, NCH_3 and NCH_2), 2.55 (2H, dd, J 12.81 Hz and J 4.21 Hz, NCH_2), 3.50 (6H, m, CHO and OCH_2CH_2),

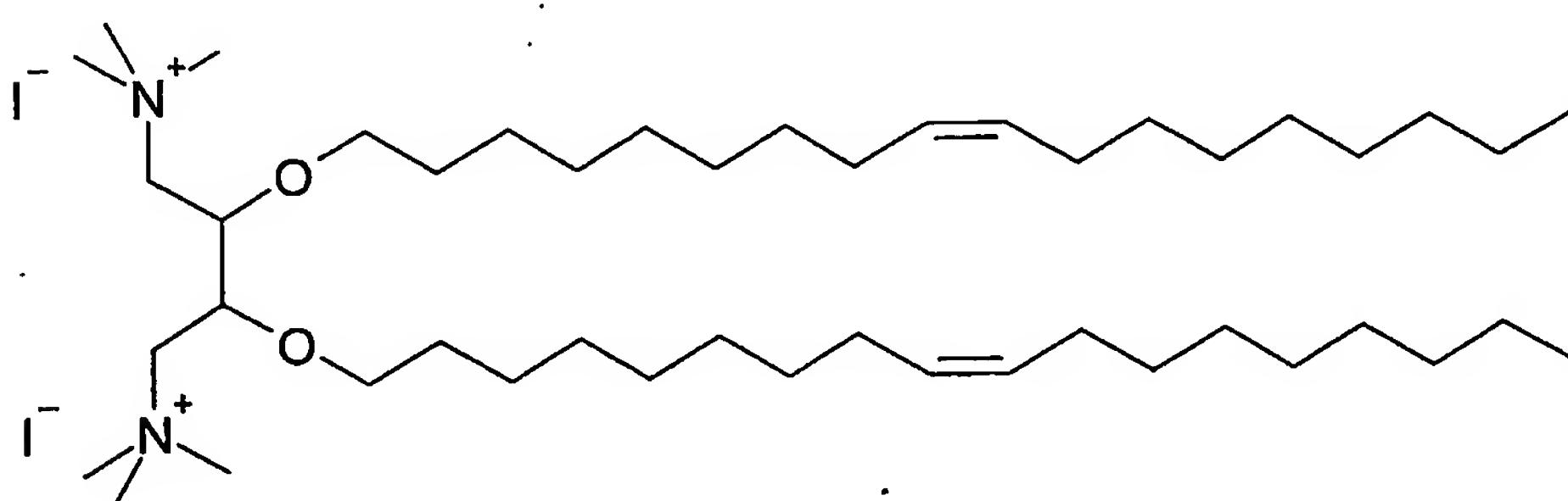
5 5.35 (4H, m, $CH=CH$);

δ_C (75 MHz, $CDCl_3$) 14.44 (CH_2CH_3), 23.04 – 32.96, 46.62 (NCH_3), 59.47 (NCH_2), 71.21 (OCH_2CH_2), 77.68 (CHO), 130.19 and 130.28 ($CH=CH$);

ν_{max} cm^{-1} (Film) 3415.7, 2925.8, 2854.5, 2767.7, 2353.0, 2239.2, 1654.8, 1461.9, 1099.3;

10

The third step produced 1,4-Di(trimethylammonium)-2,3-dioleyloxy-butane; diiodide



15 1,4-Di(dimethylamino)-2,3-dioleyloxy-butane (305 mg, 0.45 mmol) is stirred in iodomethane (2.00 ml) in a sealed tube at 90 °C for 24 hr. Excess iodomethane was removed in vacuo. The product was recrystallized (ethyl acetate) to obtain the product as a waxy solid (366 mg, 85%).

20 δ_H (300 MHz, $CDCl_3$) 0.91 (6H, t, J 6.9 Hz, CH_2CH_3), 1.25 (44H, m), 1.55 (4H, m, OCH_2CH_2), 2.00 (8H, m, $CH_2CH=CHCH_2$), 3.45 (18H, s, NCH_3), 3.75 (2H, m, OCH_2CH_2), 4.01 (6H, m, OCH_2CH_2 and NCH_2), 4.50 (2H, m, CHO), 5.35 (4H, m,

-45-

CH=CH);

δ_c (75 MHz, DEPT, CDCl₃) 14.44 (CH₂CH₃), 23.04 – 32.96, 55.93 (NCH₃), 68.26

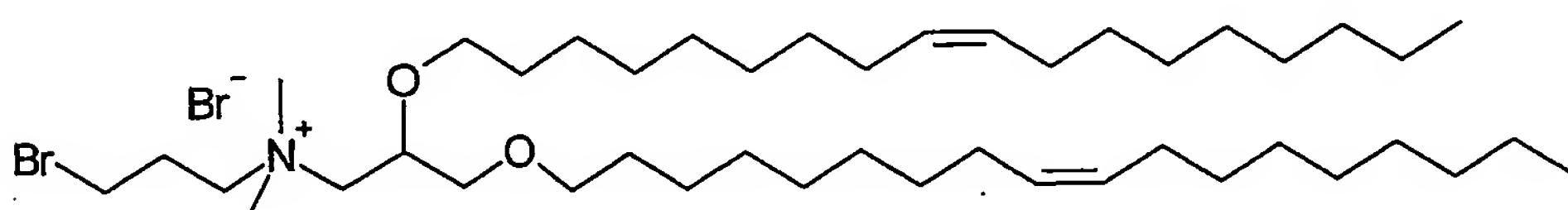
(NCH₂), 72.49 (OCH₂CH₂), 73.54 (CHO), 130.19 and 130.28 (CH=CH);

m/z (ESP+) 353.6 (100%, $1/2M^+$);

5 ν_{max} cm⁻¹ (nujol mull) 3418.6, 2916.6, 2864.6, 2718.5, 2353.9, 1625.9, 1464.8, 1376.6;

Example 3

10 Example 3 illustrates the use of Reaction Scheme 3. [2,3-Di-(oleyloxy)-propyl]-(3-bromo-propyl)-dimethyl-ammonium; bromide was prepared in a first step



15 1,2-Dioleyloxy-3-dimethylamino propane (0.50 g, 0.81 mmol) and 1,3-dibromopropane (0.82 ml, 8.10 mmol) were stirred in methanol in a sealed tube at 80 °C for 18 hr. The solvent was removed *in vacuo*. The product was purified by flash chromatography (gradient; DCM to 10% methanol in DCM) to yield the above product as a yellow oil (0.41 g, 62%).

20

R_f = 0.17 (5% methanol in DCM);

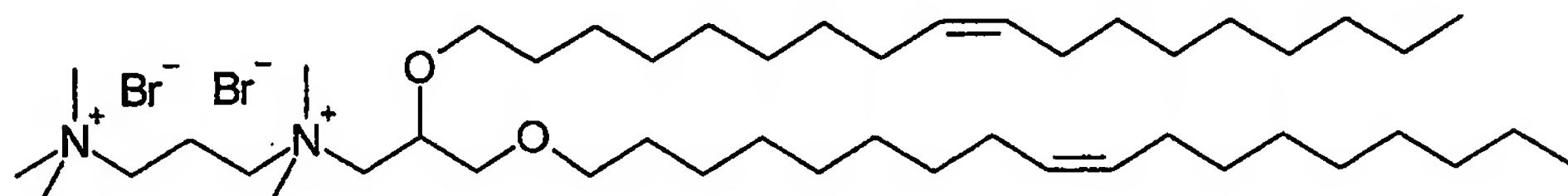
δ_H (300 MHz, CDCl₃) 0.87 (6H, t, *J* 7.0 Hz, CH₂CH₃), 1.28 (44H, m), 1.57 (4H, m, OCH₂CH₂), 2.01 (8H, m, CH₂CH=CHCH₂), 2.45 (2H, m, BrCH₂CH₂CH₂N), 3.40 – 4.10 (19H, m, CHO, CH₂O, NCH₃, BrCH₂CH₂CH₂N), 5.33 (4H, m, CH=CH);

25 δ_c (75 MHz, CDCl₃) 14.50 (CH₂CH₃), 23.00 – 33.10, 53.25 and 53.56 (NCH₃), 65.00 (NCH₂), 66.29 (NCH₂), 68.87, 69.94, 72.57 and 73.78 (CHO and CH₂O), 130.24 and 130.48 (CH=CH);

-46-

ν_{max} cm⁻¹ (Film) 3417.6, 2923.9, 2852.5, 2358.8, 1633.6, 1463.9, 1120.6;

The above product was then converted, in a second reaction step, to [2,3-Di-(oleyloxy)-propyl]-(3-trimethyl-ammonium-propyl)-dimethyl-ammonium; dibromide.



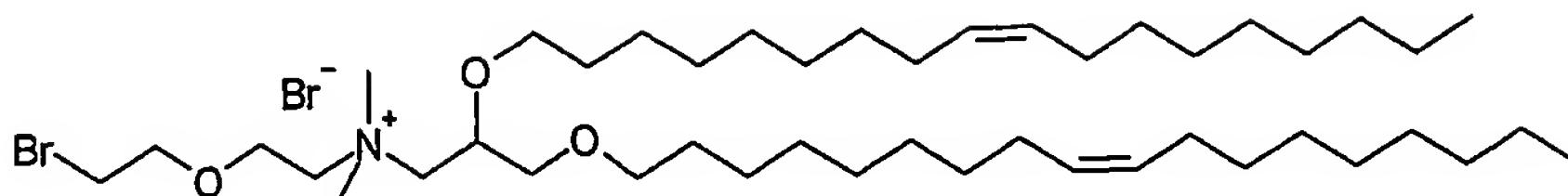
[2,3-Di-(oleyloxy)-propyl]- (3-bromo-propyl)-dimethyl-ammonium; bromide (100 mg, 0.12 mmol) and trimethylamine solution (45 wt% in H₂O, 0.094 ml, 0.61 mmol) were stirred in methanol (2 ml) in a sealed tube at 90 °C for 24 hr. The solvent was removed *in vacuo* and the resulting residue was purified by recrystallization (ethyl acetate) to yield the above product as an off white solid (86.6 mg, 82%).

15 δ_{H} (300 MHz, CDCl_3) 0.87 (6H, t, J 6.9 Hz, CH_2CH_3), 1.28 (44H, m), 1.53 (4H, m, OCH_2CH_2), 2.01 (8H, m, $\text{CH}_2\text{CH}=\text{CHCH}_2$), 2.72 (2H, m, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{N}$), 3.40 – 4.10 (28H, m, CHO, CH_2O , NCH_3 , $\text{NCH}_2\text{CH}_2\text{CH}_2\text{N}$), 5.35 (4H, m, $\text{CH}=\text{CH}$); m/z (ESP+) 360.6 (100%, ${}^1/{}_2\text{M}^+$); ν_{max} cm^{-1} (Film) 3382.4, 2922.4, 2852.0, 1655.8, 1455.7, 1116.7;

Example 4

The fourth reaction scheme is illustrated by Example 4. [2,3-Di-(oleyloxy)-propyl]-[2-(2-bromo-ethoxy)-ethyl]-dimethyl-ammonium; bromide was prepared in a first step.

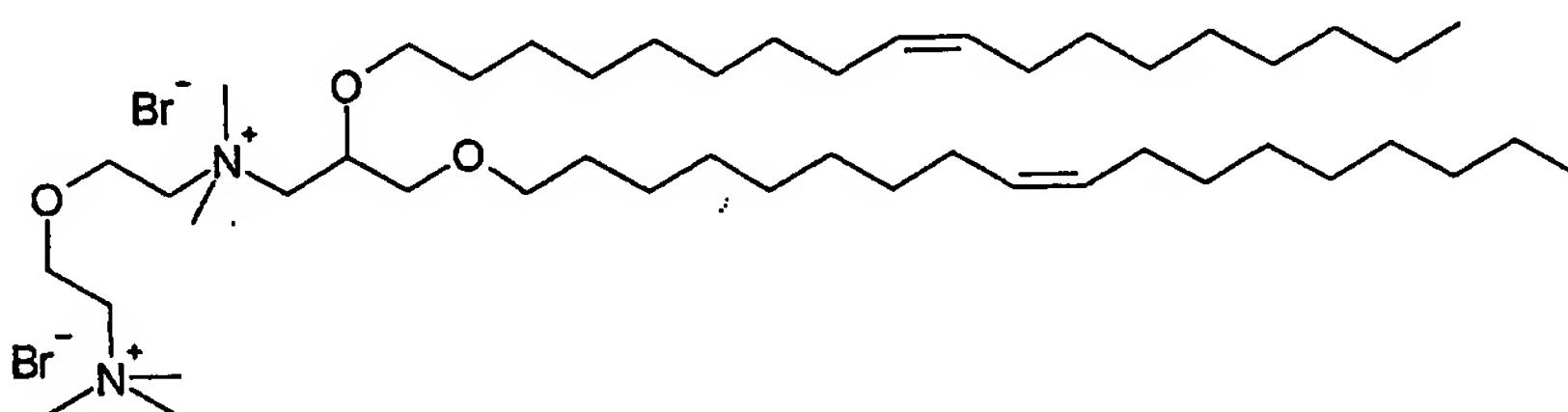
-47-



2-Bromoethyl ether (278 mg, 1.20 mmol) and 1,2-dioleyloxy-3-dimethylamino propane (300 mg, 0.48 mmol) were stirred in methanol (2 ml) at 90 °C in a sealed tube for 24 hr. The solvent was removed *in vacuo*. The product was purified by recrystallization (ethyl acetate) to yield the above product as a yellow oil at r.t. and a off white solid upon freezing (197 mg, 48%).

δ_H (300 MHz, $CDCl_3$) 0.90 (6H, t, J 7.0 Hz, CH_2CH_3), 1.25 (44H, m), 1.52 (4H, m, $OCH_2CH_2CH_2$), 2.01 (8H, m, $CH_2CH=CHCH_2$), 3.40 – 4.10 (23H, m, CH_2O , CHO , NCH_2 , NCH_3 , CH_2Br), 5.34 (4H, m, $CH=CH$);
 m/z (ESP+) 773.6 (45%, M^+), 772.6 (100%, M^+), 771.6 (45%, M^+), 770.6 (88%, M^+);
 ν_{max} cm^{-1} (Film) 3428.2, 2926.8, 2854.0, 2343.8, 1641.8, 1464.8, 1366.5, 1122.0;

In a second step, the above product was converted to [2,3-Di-(oleyloxy)-propyl]-[2-(2-trimethyl-ammonium-ethoxy)-ethyl]-dimethyl-ammonium; dibromide



[2,3-Di-(oleyloxy)-propyl]-[2-(2-bromo-ethoxy)-ethyl]-dimethyl-ammonium; bromide (100 mg, 0.12 mmol) and trimethylamine solution (45 wt% in H_2O , 0.31 ml, 2.35 mmol) in methanol (3 ml) were stirred at 90 °C in a sealed tube for 24 hr. The

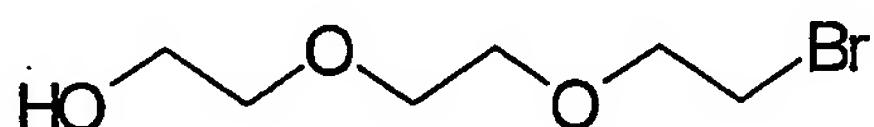
-48-

solvent was removed *in vacuo*. The residue was treated with diethyl ether and the precipitate was collected by filtration. The off white solid was the above product (73 mg, 67%).

5 δ_H (300 MHz, CDCl₃) 0.85 (6H, t, *J* 6.9 Hz, CH₂CH₃), 1.22 (44H, m), 1.50 (4H, m, OCH₂CH₂CH₂), 2.01 (8H, m, CH₂CH=CHCH₂), 3.40 – 4.10 (32H, m, CH₂O, CHO, NCH₂, NCH₃), 5.35 (4H, m, CH=CH);
10 δ_C (75 MHz, CDCl₃) 14.46 (CH₂CH₃), 23.00 – 33.00, 55.23 (NCH₃), 65.53 – 74.00, 130.26 and 130.47 (CH=CH);
m/z (ESP+) 375.6 (100%, $1/2M^+$);
 ν_{max} cm⁻¹ (Film) 3391.6, 2922.4, 2852.5, 1703.0, 1462.4, 1361.7, 1125.9;

Example 5

15 Example 5 illustrates Reaction Scheme 5. 2-[2-(2-Bromo-ethoxy)-ethoxy]-ethanol was produced in a first step.



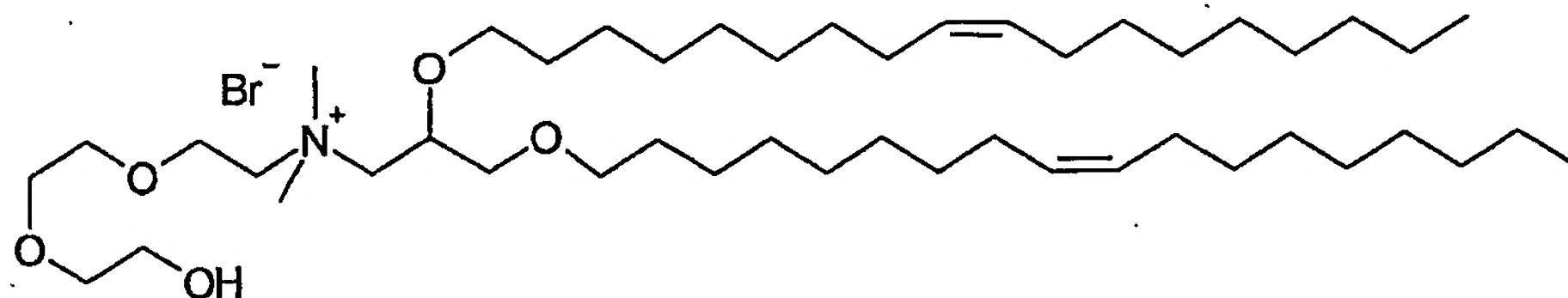
20 Tri (ethylene) glycol (4.50 g, 30.0 mmol) and hydrobromic acid solution (48%, 5.09 ml, 45.0 mmol) were stirred in toluene (70 ml) at reflux for 72 hr. After cooling the solution was neutralized by the addition of saturated sodium hydrogencarbonate solution. Water (50 ml) was added and the resulting mixture was extracted with DCM (3 x 50 ml). The chlorinated extract was dried over anhydrous magnesium sulfate and concentrated *in vacuo*. No further purification was required and the 25 above product was obtained as a yellow oil (2.29 g, 36%).

δ_H (300 MHz, CDCl₃) 2.64 (1H, s, OH), 3.42 (2H, t, *J* 6.2 Hz, CH₂Br), 3.55 – 3.65 (8H, m, CH₂O), 3.76 (2H, t, *J* 6.2 Hz, CH₂CH₂Br);
 δ_C (75 MHz, CDCl₃) 30.65 (CH₂Br), 62.05 (CH₂OH), 70.70, 71.51 and 72.93

-49-

 (CH_2O) ; $\nu_{\max} \text{cm}^{-1}$ (Film) 3415.7, 2920.0, 2871.8, 2339.5, 1641.3, 1454.2;

5 In a second step, the above product was converted to [2,3-Di-(oleyloxy)-propyl]-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethyl}-dimethyl-ammonium; bromide



10 2-[2-(2-Bromo-ethoxy)-ethoxy]-ethanol (223 mg, 0.96 mmol) and 1,2-dioleyloxy-3-dimethylamino propane (300 mg, 0.48 mmol) in methanol (2 ml) were stirred at 90 °C in a sealed tube for 24 hr. The solvent was removed *in vacuo*. The product was purified by recrystallization (ethyl acetate) to yield the above product as a pale yellow oil at r.t. and a white solid upon freezing (247 mg, 62%).

15 δ_{H} (300 MHz, CDCl_3) 0.85 (6H, t, J 6.99 Hz, CH_2CH_3), 1.25 (44H, m), 1.54 (4H, m, OCH_2CH_2), 2.01 (8H, m, $\text{CH}_2\text{CH}=\text{CHCH}_2$), 2.88 (1H, brs, OH), 3.40 – 4.10 (27H, m, CH_2O , CHO, NCH_2 , NCH_3 , CH_2OH), 5.35 (4H, m, $\text{CH}=\text{CH}$);
 m/z (ESP+) 752.7 (100%, M^+);
 $\nu_{\max} \text{cm}^{-1}$ (Film) 3371.3, 2916.6, 2854.0, 2666.4, 2333.2, 1641.8, 1464.8, 1366.5, 1117.2.

20 Example 6

Transfection Efficiencies

25 The activity of various compositions of the invention were measured in HAE cells using a Luciferase assay. RLU/mg refers to relative light units per mg of protein. The assay was carried out as follows:

-50-

Lipid (10 mg/mL; 100 μ L [1 mg of lipid]) in chloroform was placed into a glass vial (sterile). The solvents were removed *in vacuo* and further traces of chloroform were removed on the high vacuum for 24 h. Cationic Lipids were either formulated alone or with DOPE (mole ratio, 1:1).

5 Deionized water (1 mL; MilliQ) was added to the film, to generate 1 mg/mL solution of lipid in water. The mixture was allowed to hydrate at 4 °C for 24 h. After warming to 40 °C the mixture was sonicated to generate a clear solution (5 min approx). The resulting liposome formulations were stable for up to 3months.

10 The components of the complex were mixed in a the required weight ratio. The lipid was initially mixed with the peptide and the resulting mixture was added to the plasmid DNA.

15 Human airway epithelial cells were seeded for 24 h at 37 °C in complete growth medium in a 96 well plate. Transfection complexes were left to aggregate for 30 min and diluted to a concentration of 1 μ g of DNA per 0.5 ml in OptiMEM (Life technologies). The medium was removed from each well and replaced with 0.5 ml of transfection complex and was allow to incubate for a further 4 h. The transfection complexes were removed and then replace with growth medium and the cells were allowed to incubate for 48 h.

20 The transfected cells were washed with phosphate-buffered saline (PBS). Reporter lysis buffer (100 μ l) (Promega) was added to each well and cooled to 4 °C for 15 min. The cells were then assayed with a luciferase assay kit. The total light emission was measured for each well for 60 sec. The protein concentration of each sample was determined using a protein assay reagent and the activity expressed as relative light units per milligram of protein (RLU/mg).

25 The values obtained for the compositions produced in Examples 1 and 2 above and for Lipofectin were measured at 1:1 and 2:1 ratios of lipid:DNA, and in each case the ratio of peptide:DNA was 4:1, the peptide used being [K]₁₆ GACRRETAWACG. The ratios are expressed in terms of weight. The testing procedure used was that outlined in Human Gene Therapy 9, 575-585, 1998.

-51-

Example 1	(1:1, lipid:DNA) C18 Ester	1.2 RLU/mg
Example 2	(1:1, lipid:DNA) C18 Ether	43.2 RLU/mg
Lipofectin	(1:1, lipid:DNA)	12.1 RLU/mg
Example 1	(2:1, lipid:DNA) C18 Ester	2.0 RLU/mg
5 Example 2	(2:1, lipid:DNA) C18 Ether	373.3 RLU/mg
Lipofectin	(2:1, lipid:DNA)	51.6 RLU/mg

Values were also obtained for compositions produced by Reaction Scheme 3 above, in particular C18 unsaturated compounds having $(CH_2)_3$ and $(CH_2)_6$ spacer 10 groups (i.e. n in Reaction Scheme is 3 or 6) and where the terminal nitrogen carries three methyl groups. The results were as follows:

$n = 3$ 31.8 RLU/mg

$n = 6$ 19.8 RLU/mg

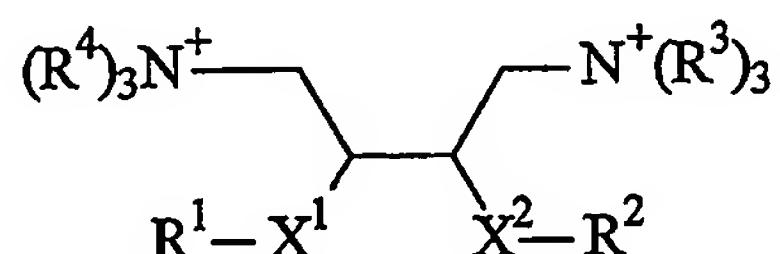
Lipofection 12.1 RLU/mg

-52-

CLAIMS

1. A complex suitable for delivery of a biologically-active material to a cell, which complex comprises:

5 (i) a lipid of general formula (I) or (II):



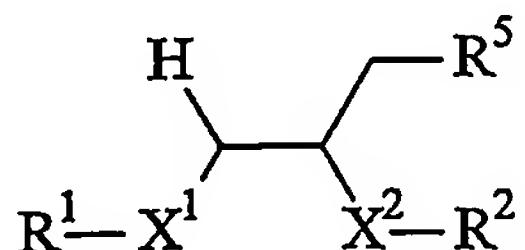
10

(I)

wherein:

- X^1 and X^2 are the same or different and are selected from $-\text{O}-\text{CH}_2-$, and
- 15 $-\text{O}-\text{C}(\text{O})-$;
- R^1 and R^2 are the same or different and are straight or branched, saturated or unsaturated C_7 to C_{24} hydrocarbyl groups which are unsubstituted or substituted by one or more substituents selected from hydroxy, halogen and OR' , wherein R' is a C_1 to C_6 hydrocarbyl group;
- 20 – each R^3 and each R^4 is the same or different and is a straight or branched, saturated or unsaturated C_1 to C_{10} hydrocarbyl group which is unsubstituted or substituted by one or more substituents selected from hydroxy, halogen, $-\text{OR}'$, $-\text{C}(\text{O})\text{OH}$, $-\text{CN}$, $-\text{NR}'\text{R}''$, and $-\text{C}(\text{O})\text{R}''$ wherein R' and R'' are the same or different and are C_1 to C_6 hydrocarbyl;

25



(II)

30

-53-

wherein:

- X^1 and X^2 are the same or different and are as defined above;
- R^1 and R^2 are the same or different and are as defined above;
- R^5 is $-N^e(R^3)_2-R^6$ wherein each R^3 is the same or different and is as defined above and R^6 is either

5

- (a) $\{A-Y\}_nR^4$ wherein
 - each Y is the same or different and is $-N^e(R^4)_2-$, wherein R^4 is as defined above,
 - each A is the same or different and is a C_1 to C_{20} alkylene group which is unsubstituted or substituted by one or more substituents selected from hydroxy, halogen, $-OR'$, $-C(O)OH$, $-CN$, $-NR'R''$, and $-C(O)R''$ wherein R' and R'' are the same or different and are C_1 to C_6 hydrocarbyl,
 - n is from 1 to 10, and
 - R^4 is as defined above; or

10

- (b) $\{B-O\}_mB-Q$ wherein
 - each B is the same or different and is a C_1 to C_{10} alkylene group which is unsubstituted or substituted by one or more substituents selected from hydroxy, halogen, $-OR'$, $-C(O)OH$, $-CN$, $-NR'R''$ and $-C(O)R''$ wherein R' and R'' are the same or different and are C_1 to C_6 hydrocarbyl,
 - m is from 1 to 10, and
 - Q is selected from $N^e(R^3)_3$, OH , OR' , $OC(O)R'$ and halogen, wherein R^3 and R' are as defined above; and

- (ii) a biologically-active material.

20

- 25 2. A complex according to claim 1, wherein X^1 and X^2 are the same.
- 3. A complex according to claim 1 or 2, wherein R^1 and R^2 are the same or different and represent a palmitic, stearic, oleic, linoleic or linolenic residue.

30

-54-

4. A complex according to any one of the preceding claims wherein each R³ and each R⁴ is unsubstituted or substituted by one or more substituents selected from hydroxy, -OR', -C(O)OH, -CN, -NR'R" and -C(O)R" wherein R' and R" are the same or different and are C₁ to C₆ hydrocarbyl.
5
5. A complex according to any of claims 1 to 4, wherein each R³ and each R⁴ are the same or different and are straight or branched, saturated or unsaturated C₁ to C₄ hydrocarbyl groups which are unsubstituted or substituted by one or two substituents selected from hydroxy and OR' wherein R' is a C₁ to C₆ alkyl group.
10
6. A complex according to any one of the preceding claims, wherein n is selected from 1 and 2.
- 15 7. A complex according to any one of the preceding claims, wherein m is selected from 1, 2 and 3.
8. A complex according to any one of the preceding claims, wherein A is an unsubstituted C₃, C₄ or C₅ alkylene group.
20
9. A complex according to any one of the preceding claims, wherein B is an unsubstituted C₂, C₃ or C₄ alkylene group.
- 25 10. A complex according to any one of the preceding claims, wherein Q is N⁺(R³)₃ or OH.
11. A complex according to any one of the preceding claims, wherein R¹ and R² are an oleic residue.
30
12. A complex according to any of claims 1 to 12, wherein R³ and R⁴ are methyl;

13. A complex according to any one of the preceding claims, wherein the biologically-active material is a nucleic acid, a peptide or a small molecule.
- 5 14. A complex according to claim 13, wherein the nucleic acid is a DNA.
15. A complex according to claim 13, wherein the nucleic acid is an RNA.
- 10 16. A complex according to any one of claims 13 to 15, wherein the nucleic acid comprises a coding sequence.
17. A complex according to any one of the preceding claims, which comprises an integrin-binding peptide.
- 15 18. A complex according to claim 17, wherein the integrin-binding peptide comprises all or part of an integrin-binding domain of a naturally-occurring integrin ligand.
19. A complex according to any one of the preceding claims which comprises a 20 polycationic component.
20. A complex according to claim 19, wherein the integrin-binding peptide and the polycationic component are coupled.
- 25 21. A complex according to any one of the preceding claims which comprises a neutral lipid.
22. A process for the preparation of a complex according to claim 1, which method comprises: admixing (i) a lipid of formula (I) or (II) as defined in claim 1; and 30 (ii) a biologically-active material.

-56-

23. A complex obtainable by a process according to claim 22.
24. A mixture comprising:
 - (i) a lipid of formula (I) or (II) as defined in any one of claims 1 to 12; and
 - 5 (ii) (a) an integrin-binding peptide; and/or
(b) a polycationic component; and/or
(c) a neutral lipid.
- 10 25. A mixture according to claim 24, wherein the integrin-binding peptide is as defined in claim 18.
- 15 26. A process for the preparation of a complex according to any one of claims 1 to 21, which process comprises admixing a mixture according to claim 24 or 25 with a biologically-active material.
- 20 27. A method for transfecting a cell with a biologically-active material, which method comprises contacting the cell *in vivo*, *in vitro* or *ex vivo* with a complex according to any one of claims 1 to 21 or 23.
- 25 28. A method for the expression of a nucleic acid in a cell, which method comprises transfecting the cell with a complex according to claim 16 using the method of claim 27 under conditions to provide for expression of the nucleic acid component of the complex.
- 30 29. A method for the preparation of a polypeptide, which method comprises:
 - (a) transfecting a cell with a complex according to claim 16 using the method of claim 27 under conditions to provide for expression of the polypeptide encoded by the nucleic acid component of the complex; and
 - (b) recovering the expressed polypeptide.

30. Use of a complex according to any one of claims 1 to 21 or 23 in transfecting a cell, in expressing a nucleic acid in a cell or in the preparation of a polypeptide.
- 5 31. A pharmaceutical composition comprising a complex according to any one of claims 1 to 21 or 23 and a pharmaceutically-acceptable carrier, diluent or excipient.
- 10 32. A complex according to any one of claims 1 to 21 or 23 for use in a method of prophylaxis or treatment of the human or animal body by therapy.
33. Use of a complex according to any one of claims 1 to 21 or 23 in the manufacture of a medicament for use in the prophylaxis or treatment of a condition caused by or related to a genetic defect or modification.
- 15 34. Use of a complex according to any one of claims 1 to 21 or 23 in the manufacture of a medicament for use in the prophylaxis or treatment of a condition by an anti-sense nucleic acid or an iRNA.
- 20 35. A method for the treatment of a condition caused by or related to a genetic defect or modification in a host, which method comprises administering to the host a therapeutically effective amount of a complex according to any one of claims 1 to 21 or 23.
- 25 36. A method for the treatment of a condition in a host by an anti-sense nucleic acid or an iRNA, which method comprises administering to the host a therapeutically effective amount of a complex according to any one of claims 1 to 21 or 23.

-58-

37. A vaccine comprising a complex according to any one of claims 1 to 21 or 23 and a pharmaceutically-acceptable carrier, diluent or excipient.

5 38. A complex according to any one of claims 1 to 21 or 23 for use in a method of vaccinating a human or animal.

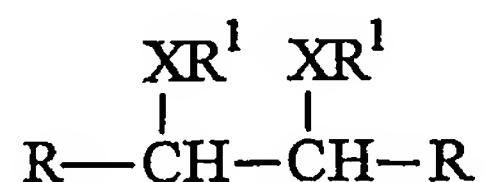
39. Use of a complex according to any one of claims 1 to 21 or 23 in the manufacture of a medicament for use in vaccinating a human or animal.

10 40. A method for raising an immune response in a mammalian host, which comprises administering to the host a complex according to any one of claims 1 to 21 or 23.

15 41. A method for modifying a cell, which method comprises contacting the cell with a complex according to any one of claims 1 to 21 or 23.

42. A lipid of formula (I) or (II) as defined in any one of claims 1 to 12.

20 43. A composition including the structure (III):



(III)

-59-

wherein:

the R_s, which may be the same or different, are

(a) H,

(b) -CH₂-N⁺(R²)₂-CH₂-CH₂[Y-(CH₂)_p]_q-Z, or

5 (c) -CH₂-N⁺(R⁴)₃,

with the proviso that one R is H and the other is group (b); or both groups R are groups (c); and wherein

the X_s which may be the same or different, are OCH₂ or O-C(O);

10 the R¹s, which may be the same or different, are saturated or unsaturated, C₇ to C₂₃ chains;

the R²s, which may be the same or different, are C₁ to C₆ saturated or unsaturated chains;

Y is NH, CH₂, O or N(acetyl);

15 Z is O(C₁ to C₄), OC(O)R³, N⁺R₃⁴, OH, F, Cl, Br or I where R³ is C₁ to C₆ alkyl;

the R⁴s, which may be the same or different, are C₁ to C₆ chains;

n is from 2, 3 or 4; and

20 m is from 1 to 200 and where it is at least 2 the resulting repeating units may be the same or different.

25

44. 1,4-Di(trimethylammonium)-2,3-dioleyloxy-butane; diiodide;
1,4-Di(trimethylammonium)-2,3-dioleyloxy-butane; diiodide;
[2,3-Di-(oleyloxy)-propyl]-[3-trimethyl-ammonium-propyl]-dimethyl-
ammonium; dibromide;
[2,3-Di-(oleyloxy)-propyl]-[2-(2-trimethyl-ammonium-ethoxy)-ethyl]-
dimethyl-ammonium; dibromide; and
[2,3-Di-(oleyloxy)-propyl]-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethyl}-
dimethyl-ammonium; bromide.

30

INTERNATIONAL SEARCH REPORT

PCT/GB 03/01985

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, MEDLINE, BIOSIS, CHEM ABS Data, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 824 812 A (BALASUBRAMANIAM RAJIV P ET AL) 20 October 1998 (1998-10-20) column 12; claim 21; figure 4 ---	1-5, 11-16, 19, 22, 23, 27-43
A	US 5 869 715 A (BALASUBRAMANIAM RAJIV P ET AL) 9 February 1999 (1999-02-09) the whole document ---	1-44
A	HART S L ET AL: "Lipid -mediated enhancement of transfection by a nonviral integrin -targeting vector" HUMAN GENE THERAPY, vol. 9, 1 March 1998 (1998-03-01), pages 575-585, XP002080439 ISSN: 1043-0342 --- -/-	

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the International search

22 August 2003

Date of mailing of the International search report

11.09.03

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Stoyanov, B

INTERNATIONAL SEARCH REPORT

PCT/GB 03/01985

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 00 57917 A (VICAL INC ;WHEELER CARL J (US)) 5 October 2000 (2000-10-05) the whole document ----	
A	WO 00 73263 A (VICAL INC ;WHEELER CARL J (US)) 7 December 2000 (2000-12-07) the whole document ----	

INTERNATIONAL SEARCH REPORT

PCT/GB 03/01985

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 27-28, 30, 35-36, 40-41 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-5, 11-44 (only partially)

A complex comprising the lipid of general formula I and the uses thereof

**2. Claims: claims 1-5,
11-44 (only partially); claims 6-10 (completely)**

A complex comprising the lipid of general formula II and the uses thereof

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claim 43 contains undefined subject matter (see e.g. indexes "p" and "q" in claim 43(b) and is therefore completely unclear.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

PCT/GB 03/01985

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
US 5824812	A	20-10-1998		US 5869715 A		09-02-1999
				AU 7078396 A		30-04-1997
				AU 713093 B2		25-11-1999
				AU 7372996 A		17-04-1997
				CA 2233064 A1		03-04-1997
				EP 1019361 A1		19-07-2000
				JP 2002515024 T		21-05-2002
				WO 9711935 A1		03-04-1997
				WO 9713743 A1		17-04-1997
				US 5925623 A		20-07-1999
US 5869715	A	09-02-1999		AU 7078396 A		30-04-1997
				AU 713093 B2		25-11-1999
				AU 7372996 A		17-04-1997
				CA 2233064 A1		03-04-1997
				EP 1019361 A1		19-07-2000
				JP 2002515024 T		21-05-2002
				WO 9713743 A1		17-04-1997
				WO 9711935 A1		03-04-1997
				US 5925623 A		20-07-1999
				US 5824812 A		20-10-1998
WO 0057917	A	05-10-2000		CA 2365416 A1		05-10-2000
				EP 1165140 A2		02-01-2002
				JP 2002540173 T		26-11-2002
				WO 0057917 A2		05-10-2000
				US 6586409 B1		01-07-2003
WO 0073263	A	07-12-2000		CA 2375727 A1		07-12-2000
				EP 1183231 A1		06-03-2002
				JP 2003500469 T		07-01-2003
				WO 0073263 A1		07-12-2000